I. Basis

The documents mentioned in the present written opinion / International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

Claims 31-36 have not been examined since they are considered to contain subject-matter extending beyond that disclosed in the application documents as originally filed. These claims refer to hydroxy residues at the C-5 position of the monoligol ring and enzymes enabling hydroxylation at this position. This cannot be found in application documents as originally filed.

II. Priority

The filed application document contains minor but significant changes from the priority document. Whereas priority document provides for modification of monoligol residues and preferably a greater content of syringyl residues, the filed document adds or other residues with a side group at the C-5 position of the monoligol ring. Any matter relating thereto is not entitled to priority.

III. No Opinion

Claims 31-36 since contain unallowable added matter (see section I).

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

- Novelty (Art.33(2) PCT)

D1 discloses coniferin beta-galactosidase (CBG) for use in modifying lignin content in plants. Either express additional enzyme to increase lignin biosynthesis or express antisense to inhibit it. CBG hydrolyzes coniferin and syringin to coniferyl and sinapyl alcohols. This then enables sinapyl alcohol production in plants where the further requisite enzymes are present. Thus, regulation of said activity increases or decreases the general level of lignins in the plant. The transformed plant can be a conifer such as Pinus sp. D1 anticipates claims 1-4,

8-12, 15-17, 22, 24, 25, 28-30.

D2 and D3 effectively both disclose the present invention. Both suggest the transgenic expression of F5H enzyme in gymnosperms, particularly of Pinus and Picea sp., in order to increase syringyl monomer levels in the lignin polymer. Since these monomers cannot crosslink, a better resource is provided for the pulping industry. These documents thus anticipate claims 1-30.

It is noted that the examples of D2 and D3 do not use gymnosperms. However, gymnosperm transformation is a routine procedure and thus putting teaching of D2 or D3 into practice cannot have been considered to involve overcoming a significant technical hurdle. Applicant confirms this since he applied standard gymnosperm transformation technology.

D4 provides a similar disclosure to that of D2 and D3, but is broader in scope since it covers manipulation of several (individually named) lignin biosynthesis genes including F5H (for which it provides F5H from Eucalyptus grandis). D4 is also novelty-destroying for claims 1-30.

D5 suggests that F5H regulation could be interesting for lignin modification. However, not cited against novelty of claims.

Inventive Step (Art.33(3) PCT)

None of present claims even novel, so question of inventivity not raised at present.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

VI. **Certain documents**

In accordance with Rule 70.10, PCT, applicants attention is drawn to the following document(s):

D8: WO-A-99/31243 (Publication date, 24.06.99; Priority date, 16.12.97; Filing date, 16.12.98)

VIII. **Certain observations**

- **Clarity (Art.6 PCT)**

Claim 2 - the F5H gene used in the invention can hardly be said to be required for the biosynthesis of lignin in the gymnosperm plant. Lignin can be biosynthesized without the gene.

Claim 5, 18 - terminology "substantially homologous activity" is technically meaningless.

Claim 6, 7, 19, 20 - "homology" is unclear term unlike "identity". Also length over which homology is observed is not specified.

Claim 28 - wording i.e. plant A cannot "have" plant B

CLAIMS:-

1. A process of producing a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild type plants of the same gymnosperm species, which process comprises:

providing a vector containing at least one expressible transgene that results in modification of the lignin composition in the gymnosperm plant;

introducing said vector into cells of a gymnosperm to produce transformed cells;

regenerating transformed gymnosperm callus or shoots from the transformed cells;

maturing embryos from the transformed callus or shoots; and

generating transformed plant embryos, seeds, seedlings or plants from the matured embryos or shoots.

2. A process according to claim 1, characterized in that said vector is provided with said at least one expressible transgene that encodes at least one enzyme required for the biosynthesis of lignin in the gymnosperm plant.

3. A process according to claim 1, characterized in that said vector is provided with said expressible transgene that encodes at least one enzyme enabling the production of sinapyl alcohol or other residues with a side group at the C-5 position of a monolignol ring during the biosynthesis of lignin.

4. A process according to claim 1, characterized in that said vector is provided with said at least one expressible transgene that encodes at least one enzyme enabling the production of lignin containing syringyl residues or other residues with a side group at the C-5 position of a monolignol ring.

5. A process according to claim 4, characterized in that said vector is provided with an expressible transgene encoding ferulate 5-hydroxylase, or a transgene that has substantially homologous activity to said ferulate 5-hydroxylase gene, either alone or in conjunction with other genes involved in lignin biosynthesis.

6. A process according to claim 5, characterized in that one of the said substantially homologous gene has at least 50% homology with said ferulate 5-hydroxylase gene.

7. A process according to claim 5, characterized in that said substantially homologous gene has at least 75% homology with said ferulate 5-hydroxylase gene.

8. A process according to any preceding claim, characterized in that said gymnosperm plant is from the order coniferales.

9. A process according to any preceding claim, characterized in that said gymnosperm plant is from the species *Picea*.

10. A process according to claim 9, characterized in that said plant is *Picea glauca, Picea sitchesis*, or *Picea engelmanii*.

11. A process according to any one of claims 1 to 8, characterized in that said gymnosperm plant is from the species *Pinus*.

12. A process according to claim 11, characterized in that said gymnosperm plant is *Pinus taeda* or *Pinus radiata*.

13. A process according to claim 5, characterized in that said ferulate 5-hydroxylase gene is operably linked with at least one regulatory sequence.

14. A process according to claim 13, characterized in that said regulatory sequence is cauliflower mosaic virus 35S promoter, a promoter for a phenylalanine ammonia lyase gene, a promoter for a p-coumaryl CoA ligase gene, a promoter for cinnamate 4-hydroxylase, or another plant promoter capable of controlling expression of plant genes.

15. A transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild type plants of the same gymnosperm species.

16. A gymnosperm plant or plant precursor according to claim 15, characterized in that said plant has a genome containing at least one expressible transgene that encodes at least one enzyme enabling the production of sinapyl alcohol, or other residue with a side group at the C-5 position of a monolignol ring, during the biosynthesis of lignin.

17. A gymnosperm plant or precursor according to claim 15, characterized in that the plant or plant precursor has a genome containing an

expressible transgene that results in a lignin composition containing syringyl residues, or other residue with a side group at the C-5 position of a monolignol ring.

18. A gymnosperm plant or precursor according to claim 15, characterized in that said at least one expressible transgene is a gene encoding ferulate 5-hydroxylase, or a transgene that has substantially homologous activity to said ferulate 5-hydroxylase gene, either alone or in conjunction with other genes involved in lignin biosynthesis.

19. A gymnosperm plant or precursor according to claim 18, characterized in that said substantially homologous gene has at least 50% homology with said ferulate 5-hydroxylase gene.

20. A gymnosperm plant or precursor according to claim 18, characterized in that said substantially homologous gene has at least 75% homology with said ferulate 5-hydroxylase gene.

21. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the order coniferales.

22. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the species *Picea*.

23. A gymnosperm plant or precursor according to claim 22, characterized in that said plant is *Picea glauca, Picea sitchesis*, or *Picea engelmanii*.

24. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the species *Pinus*.

25. A gymnosperm plant or precursor according to claim 24, characterized in that said gymnosperm plant is *Pinus taeda* or *Pinus radiata*.

26. A gymnosperm plant or precursor according to claim 18, characterized in that said ferulate 5-hydroxylase gene is operably linked with at least one regulatory sequence.

27. A gymnosperm plant or precursor according to claim 26, characterized in that said regulatory sequence is a cauliflower mosaic virus 35S promoter, a promoter for a phenylalanine ammonia lyase gene, a promoter for a p-coumaryl CoA ligase gene, a promoter for cinnamate 4-hydroxylase, or any other plant promoter capable of controlling expression of plant genes.

28. A biomass derived from a genetically transformed gymnosperm plant, said biomass containing lignin having syringyl residues, or other residue with a side group at the C-5 position of a monolignol ring, and said transformed plant having an untransformed natural wild-type plant whose lignin contains no syringyl residues, or corresponding other residues with a side group at the C-5 position of a monolignol ring.

29. A biomass according to claim 28, resulting from growing and harvesting a genetically transformed plant or plant precursor as defined in any one of claims 15 to 27.

30. A method of producing a cellulose-containing pulp useful for papermaking and the like, which comprises finely dividing a lignin-containing biomass derived from a gymnosperm plant to produce pulped mass containing lignin, and removing at least some of said

lignin from said pulped mass, characterized in that said gymnosperm plant is a genetically transformed plant producing lignin containing syringyl residues or other residues with a side group at the C-5 position of a monolignol ring.

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-18	as originally filed

Claims, No.:

1-30	as originally filed
31-36	as amended under Article 19

Drawings, sheets:

1/3-3/3	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:

II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

- ☐ the entire international application,
- ☒ claims Nos. 31-36,

because:

- ☒ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
  see separate sheet
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrialapplicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-30
Inventive step (IS)	Claims	1-30
Industrial applicability (IA)	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

PCT REQUEST

41193-PT

Original (for **SUBMISSION**) - printed on 31.01.2000 04:07:44 PM

0 **0-1**	**For receiving Office use only** International Application No.	PCT / CA 00/00074
0-2	International Filing Date	31 JANUARY 2000 (31.01.00)
0-3	Name of receiving Office and "PCT International Application"	RO/CA
0-4 0-4-1	**Form - PCT/RO/101 PCT Request** Prepared using	PCT-EASY Version 2.90 (updated 15.12.1999)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	Canadian Patent Office (RO/CA)
0-7	**Applicant's or agent's file reference**	41193-PT
I	**Title of invention**	MODIFICATION OF LIGNIN COMPOSITION OF GYMNOSPERMS
II	**Applicant**	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	SILVAGEN INC.
II-5	Address:	BC Research and Innovation Complex 3650 Wesbrook Mall Vancouver, British Columbia V6S 2L2 Canada
II-6	State of nationality	CA
II-7	State of residence	CA
II-8	Telephone No.	(604)221-9893
II-9	Facsimile No.	(604)224-0540
III-1	**Applicant and/or inventor**	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	ELLIS, David, Dunham
III-1-5	Address:	4887 5th Avenue Tsawwassen, British Columbia V4M 1J5 Canada
III-1-6	State of nationality	CA
III-1-7	State of residence	CA

PCT REQUEST

Original (for **SUBMISSION**) - printed on 31.01.2000 04:07:44 PM

III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	CHAPPLE, Clinton, Charles, Spencer
III-2-5	Address:	2210 Robinhood Lane West Lafayette, IN 47906-5029 United States of America
III-2-6	State of nationality	CA
III-2-7	State of residence	US
III-3	**Applicant and/or inventor**	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	GILBERT, Margarita
III-3-5	Address:	#38-23085 118 Avenue Maple Ridge, British Columbia V2X 3J7 Canada
III-3-6	State of nationality	CA
III-3-7	State of residence	CA
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	GALE, Edwin, J.
IV-1-2	Address:	Kibry, Eades, Gale, Baker Box 3432, Station D Ottawa, Ontario K1P 6N9 Canada
IV-1-3	Telephone No.	(613)237-6900
IV-1-4	Facsimile No.	(613)237-0045
IV-1-5	e-mail	email@kirbyeades.com
IV-2	**Additional agent(s)**	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	EADES, Norris, M.; BAKER, John, A.; LACHAINE, Kimberley, A.; FEUTLINSKE, Robert, K.; BAUER-MOORE, Andrew

PCT REQUEST 41193-PT

Original (for **SUBMISSION**) - printed on 31.01.2000 04:07:44 PM

V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AL AM AT AU AZ BA BB BG BR BY CA CH&LI CN CR CU CZ DE DK DM EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW
V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	**Exclusion(s) from precautionary designations**	NONE
VI-1	**Priority claim of earlier national application**	
VI-1-1	Filing date	01 February 1999 (01.02.1999)
VI-1-2	Number	60/118,124
VI-1-3	Country	US
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)

PCT REQUEST

41193-PT

Original (for **SUBMISSION**) - printed on 31.01.2000 04:07:44 PM

VIII	Check list	number of sheets	electronic file(s) attached
VIII-1	Request	4	-
VIII-2	Description	18	-
VIII-3	Claims	6	-
VIII-4	Abstract	1	41193pta.txt
VIII-5	Drawings	3	-
VIII-7	TOTAL	32	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	**Figure of the drawings which should accompany the abstract**	1	
VIII-19	**Language of filing of the international application**	English	
IX-1	**Signature of applicant or agent**		
IX-1-1	Name (LAST, First)	GALE, Edwin, J.	

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	31 JANUARY 2000 (31.01.00)
10-2	**Drawings:**	
10-2-1	Received X	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP
10-6	**Transmittal of search copy delayed until search fee is paid** X	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	

PCT (ANNEX - FEE CALCULATION SHEET)

41193-PT

Original (for **SUBMISSION**) - printed on 31.01.2000 04:07:44 PM

(This sheet is not part of and does not count as a sheet of the international application)

0 0-1	**For receiving Office use only** International Application No.	PCT / CA 00/00074
0-2	Date stamp of the receiving Office	31 JANUARY 2000 (31.01.00)

0-4 0-4-1	**Form - PCT/RO/101 (Annex)** **PCT Fee Calculation Sheet** Prepared using		PCT-EASY Version 2.90 (updated 15.12.1999)		
0-9	**Applicant's or agent's file reference**		41193-PT		
2	**Applicant**		SILVAGEN INC., et al.		
12	**Calculation of prescribed fees**		fee amount/multiplier	total amounts (CAD)	
12-1	Transmittal fee	T	⇨	200	
12-2	Search fee	S	⇨	1,450	
12-3	International fee Basic fee (first 30 sheets)	b1	630		
12-4	Remaining sheets		2		
12-5	Additional amount	(X)	15		
12-6	Total additional amount	b2	30		
12-7	b1 + b2 =	B	660		
12-8	Designation fees Number of designations contained in international application		83		
12-9	Number of designation fees payable (maximum 8)		8		
12-10	Amount of designation fee	(X)	136		
12-11	Total designation fees	D	1,088		
12-12	PCT-EASY fee reduction	R	-194		
12-13	Total International fee (B+D-R)	I	⇨	1,554	
12-17	**TOTAL FEES PAYABLE (T+S+I+P)**		⇨	3,204	
12-19	**Mode of payment**		other: NO FEES PAID AT THIS TIME		

VALIDATION LOG AND REMARKS

13-2-6	**Validation messages** **Contents**	Yellow! The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.
		Green? Priority 1. The priority document is not enclosed. (The applicant must furnish it within 16 months from the earliest priority date claimed)

ENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
41193-PT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00074	31/01/2000	01/02/1999
Applicant		
SILVAGEN INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.

   [ ] None of the figures.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/NZ 97/00112

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9315599 A		EP 0625005 A	23-11-1994
		JP 7503374 T	13-04-1995
WO 9324638 A	09-12-1993	AU 672410 B	03-10-1996
		AU 4334593 A	30-12-1993
		CA 2136618 A	09-12-1993
		EP 0643774 A	22-03-1995
		US 5633439 A	27-05-1997
WO 9620595 A	11-07-1996	AU 4744296 A	24-07-1996
		CA 2208753 A	11-07-1996
		EP 0800345 A	15-10-1997
		PL 321179 A	24-11-1997
WO 9745549 A	04-12-1997	FR 2749322 A	05-12-1997
		AU 3097297 A	05-01-1998

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
41193-PT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00074	31/01/2000	01/02/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant CELLFOR INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/08/2000	22.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00074

I. Basis of th report

1. With regard to the **lem nts** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-18	as originally filed				

Claims, No.:

1-36	as received on	15/03/2001	with letter of	15/03/2001	

Drawings, sheets:

1/3-3/3	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-36
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-36
Industrial applicability (IA)	Yes:	Claims	1-36
	No:	Claims	

2. Citations and explanations
   see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

II. Priority

The filed application document contains minor but significant changes from the priority document. Whereas priority document provides for modification of monoligol residues and preferably a greater content of syringyl residues, the filed document adds or other residues with a side group at the C-5 position of the monoligol ring. Any matter relating thereto is not entitled to priority. The fact that the application documents contain vague statements relating to modifications of monolignols or to the use of any genes that make plants more commercially desirable is not sufficient basis. These statements do not equate to the underlined matter - the underlined matter is a selection from these broader concepts.

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

The documents mentioned in the present International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

- Novelty (Art.33(2) PCT)

D1 discloses coniferin beta-galactosidase (CBG) for use in modifying lignin content in plants. Either express additional enzyme to increase lignin biosynthesis or express antisense to inhibit it. CBG hydrolyzes coniferin and syringin to coniferyl and sinapyl alcohols. This then enables sinapyl alcohol production in plants where the further requisite enzymes are present. Thus, regulation of said activity increases or decreases the general level of lignins in the plant. The transformed plant can be a conifer such as Pinus sp. D1 anticipates claims 1-4, 8-12, 15-17, 22, 24, 25, 28-30. The terminology "modification of the lignin composition" is considered to include modification of either levels of lignin or the chemical composition thereof. It is due to the former possible interpretation that the novelty objection is raised.

D2 and D3 effectively both disclose the present invention. Both suggest the transgenic expression of F5H enzyme in gymnosperms, particularly of Pinus and

Picea sp., in order to increase syringyl monomer levels in the lignin polymer. Since these monomers cannot crosslink, a better resource is provided for the pulping industry. These documents thus anticipate claims 1-36. D2 and D3 are considered enabling. It is noted that the examples of D2 and D3 do not use gymnosperms. However, gymnosperm transformation is a routine procedure and thus putting teaching of D2 or D3 into practice cannot have been considered to involve overcoming a significant technical hurdle. Applicant confirms this since he applied standard gymnosperm transformation technology. The fact that only few laboratories work on conifer transformation does not imply that the process is particularly difficult, it merely implies that a limited number of groups are interested in working in this field. The fact that D2 and D3 did not provide working examples does not mean that the disclosures are not enabling.

D4 provides a similar disclosure to that of D2 and D3, but is broader in scope since it covers manipulation of several (individually named) lignin biosynthesis genes including F5H (for which it provides F5H from Eucalyptus grandis). D4 is also novelty-destroying for claims 1-36. Indeed, it is noted that D4 covers matter extending beyond F5H in the more broadly drawn claims of the present application.

D5 suggests that F5H regulation could be interesting for lignin modification. However, not cited against novelty of claims.

Inventive Step (Art.33(3) PCT)

None of present claims even novel, so question of inventivity not raised at present.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

VI. **Certain documents**

In accordance with Rule 70.10, PCT, applicants attention is drawn to the following

document(s):
D8: WO-A-99/31243 (Publication date, 24.06.99; Priority date, 16.12.97; Filing date, 16.12.98)

VIII. **Certain observations**

- **Clarity (Art.6 PCT)**

The present number of independent claims is unacceptable

Claim 2 - the F5H gene used in the invention can hardly be said to be required for the biosynthesis of lignin in the gymnosperm plant. Lignin can be biosynthesized without the gene.

Claim 5, 18 - terminology "substantially homologous activity" was technically meaningless. New terminology "substantially equivalent function" is no clearer since the function in question is still not defined.

Claim 6, 7, 19, 20 - "homology" is unclear term unlike "identity". Also length over which homology is observed is not specified. Fact that Cambridge dictionary of biology specifies "homology" as being either (i) derived from common evolutionary origin, or (ii) reference to degree of similarity is irrelevant. Degree of similarity is not degree of identity !

Claim 28 - wording i.e. plant A cannot "have" plant B

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

GALE, Edwin J.
KIRBY, EADES, GALE, BAKER
Box 3432, Station D
Ottawa, Ontario, K1P 6N9
CANADA

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	22.05.2001
Applicant's or agent's file reference 41193-PT	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00074	31/01/2000	01/02/1999

Applicant
CELLFOR INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	CLEERE, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
41193-PT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00074	31/01/2000	01/02/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant CELLFOR INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/08/2000	22.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554

Form PCT/IPEA/409 (cover sheet) (January 1994)

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-18	as originally filed

Claims, No.:

1-36	as received on	15/03/2001	with letter of	15/03/2001

Drawings, sheets:

1/3-3/3	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
see separate sheet

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-36
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-36
Industrial applicability (IA)	Yes:	Claims	1-36
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

II. Priority

The filed application document contains minor but significant changes from the priority document. Whereas priority document provides for modification of monoligol residues and preferably a greater content of syringyl residues, the filed document adds or other residues with a side group at the C-5 position of the monoligol ring. Any matter relating thereto is not entitled to priority. The fact that the application documents contain vague statements relating to modifications of monolignols or to the use of any genes that make plants more commercially desirable is not sufficient basis. These statements do not equate to the underlined matter - the underlined matter is a selection from these broader concepts.

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

The documents mentioned in the present International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

- **Novelty (Art.33(2) PCT)**

D1 discloses coniferin beta-galactosidase (CBG) for use in modifying lignin content in plants. Either express additional enzyme to increase lignin biosynthesis or express antisense to inhibit it. CBG hydrolyzes coniferin and syringin to coniferyl and sinapyl alcohols. This then enables sinapyl alcohol production in plants where the further requisite enzymes are present. Thus, regulation of said activity increases or decreases the general level of lignins in the plant. The transformed plant can be a conifer such as Pinus sp. D1 anticipates claims 1-4, 8-12, 15-17, 22, 24, 25, 28-30. The terminology "modification of the lignin composition" is considered to include modification of either levels of lignin or the chemical composition thereof. It is due to the former possible interpretation that the novelty objection is raised.

D2 and D3 effectively both disclose the present invention. Both suggest the transgenic expression of F5H enzyme in gymnosperms, particularly of Pinus and

Picea sp., in order to increase syringyl monomer levels in the lignin polymer. Since these monomers cannot crosslink, a better resource is provided for the pulping industry. These documents thus anticipate claims 1-36. D2 and D3 are considered enabling. It is noted that the examples of D2 and D3 do not use gymnosperms. However, gymnosperm transformation is a routine procedure and thus putting teaching of D2 or D3 into practice cannot have been considered to involve overcoming a significant technical hurdle. Applicant confirms this since he applied standard gymnosperm transformation technology. The fact that only few laboratories work on conifer transformation does not imply that the process is particularly difficult, it merely implies that a limited number of groups are interested in working in this field. The fact that D2 and D3 did not provide working examples does not mean that the disclosures are not enabling.

D4 provides a similar disclosure to that of D2 and D3, but is broader in scope since it covers manipulation of several (individually named) lignin biosynthesis genes including F5H (for which it provides F5H from Eucalyptus grandis). D4 is also novelty-destroying for claims 1-36. Indeed, it is noted that D4 covers matter extending beyond F5H in the more broadly drawn claims of the present application.

D5 suggests that F5H regulation could be interesting for lignin modification. However, not cited against novelty of claims.

Inventive Step (Art.33(3) PCT)

None of present claims even novel, so question of inventivity not raised at present.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

VI. Certain documents

In accordance with Rule 70.10, PCT, applicants attention is drawn to the following

document(s):
D8: WO-A-99/31243 (Publication date, 24.06.99; Priority date, 16.12.97; Filing date, 16.12.98)

VIII. **Certain observations**

- **Clarity (Art.6 PCT)**

The present number of independent claims is unacceptable

Claim 2 - the F5H gene used in the invention can hardly be said to be required for the biosynthesis of lignin in the gymnosperm plant. Lignin can be biosynthesized without the gene.

Claim 5, 18 - terminology "substantially homologous activity" was technically meaningless. New terminology "substantially equivalent function" is no clearer since the function in question is still not defined.

Claim 6, 7, 19, 20 - "homology" is unclear term unlike "identity". Also length over which homology is observed is not specified. Fact that Cambridge dictionary of biology specifies "homology" as being either (i) derived from common evolutionary origin, or (ii) reference to degree of similarity is irrelevant. Degree of similarity is not degree of identity !

Claim 28 - wording i.e. plant A cannot "have" plant B

CLAIMS:

1. A process of producing a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild type plants of the same gymnosperm species, which process comprises:

providing a vector containing at least one expressible transgene that results in modification of the lignin composition in the gymnosperm plant;

introducing said vector into cells of a gymnosperm to produce transformed cells;

regenerating transformed gymnosperm callus or shoots from the transformed cells;

maturing embryos from the transformed callus or shoots; and

generating transformed plant embryos, seeds, seedlings or plants from the matured embryos or shoots.

2. A process according to claim 1, characterized in that said vector is provided with said at least one expressible transgene that encodes at least one enzyme affecting the phenylpropanoid pathway leading to the synthesis of lignin.

3. A process according to claim 1, characterized in that said vector is provided with said expressible transgene that encodes at least one enzyme enabling the production of sinapyl alcohol or other residues with a side group at the C-5 position of a monolignol ring during the biosynthesis of lignin.

4. A process according to claim 1, characterized in that said vector is provided with said at least one expressible transgene that encodes at least one enzyme enabling the production of lignin containing syringyl residues or other residues with a side group at the C-5 position of a monolignol ring.

5. A process according to claim 4, characterized in that said vector is provided with an expressible transgene encoding ferulate 5-hydroxylase, or a transgene that has substantially equivalent function to said ferulate 5-hydroxylase gene, either alone or in conjunction with other genes involved in lignin biosynthesis.

6. A process according to claim 5, characterized in that one of the said substantially homologous gene has at least 50% homology with said ferulate 5-hydroxylase gene.

7. A process according to claim 5, characterized in that said substantially homologous gene has at least 75% homology with said ferulate 5-hydroxylase gene.

8. A process according to any preceding claim, characterized in that said gymnosperm plant is from the order coniferales.

9. A process according to any preceding claim, characterized in that said gymnosperm plant is from the species *Picea*.

10. A process according to claim 9, characterized in that said plant is *Picea glauca, Picea sitchesis*, or *Picea engelmanii*.

11. A process according to any one of claims 1 to 8, characterized in that said gymnosperm plant is from the species *Pinus*.

12. A process according to claim 11, characterized in that said gymnosperm plant is *Pinus taeda* or *Pinus radiata*.

13. A process according to claim 5, characterized in that said ferulate 5-hydroxylase gene is operably linked with at least one regulatory sequence.

14. A process according to claim 13, characterized in that said regulatory sequence is cauliflower mosaic virus 35S promoter, a promoter for a phenylalanine ammonia lyase gene, a promoter for a p-coumaryl CoA ligase gene, a promoter for cinnamate 4-hydroxylase, or another plant promoter capable of controlling expression of plant genes.

15. A transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild type plants of the same gymnosperm species.

16. A gymnosperm plant or plant precursor according to claim 15, characterized in that said plant has a genome containing at least one expressible transgene that encodes at least one enzyme enabling the production of sinapyl alcohol, or other residue with a side group at the C-5 position of a monolignol ring, during the biosynthesis of lignin.

17. A gymnosperm plant or precursor according to claim 15, characterized in that the plant or plant precursor has a genome containing an

expressible transgene that results in a lignin composition containing syringyl residues, or other residue with a side group at the C-5 position of a monolignol ring.

18. A gymnosperm plant or precursor according to claim 15, characterized in that said at least one expressible transgene is a gene encoding ferulate 5-hydroxylase, or a transgene that has substantially equivalent function to said ferulate 5-hydroxylase gene, either alone or in conjunction with other genes involved in lignin biosynthesis.

19. A gymnosperm plant or precursor according to claim 18, characterized in that said substantially homologous gene has at least 50% homology with said ferulate 5-hydroxylase gene.

20. A gymnosperm plant or precursor according to claim 18, characterized in that said substantially homologous gene has at least 75% homology with said ferulate 5-hydroxylase gene.

21. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the order coniferales.

22. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the species *Picea*.

23. A gymnosperm plant or precursor according to claim 22, characterized in that said plant is *Picea glauca, Picea sitchesis*, or *Picea engelmanii*.

24. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the species *Pinus*.

25. A gymnosperm plant or precursor according to claim 24, characterized in that said gymnosperm plant is *Pinus taeda* or *Pinus radiata*.

26. A gymnosperm plant or precursor according to claim 18, characterized in that said ferulate 5-hydroxylase gene is operably linked with at least one regulatory sequence.

27. A gymnosperm plant or precursor according to claim 26, characterized in that said regulatory sequence is a cauliflower mosaic virus 35S promoter, a promoter for a phenylalanine ammonia lyase gene, a promoter for a p-coumaryl CoA ligase gene, a promoter for cinnamate 4-hydroxylase, or any other plant promoter capable of controlling expression of plant genes.

28. A biomass derived from a genetically transformed gymnosperm plant, said biomass containing lignin having syringyl residues, or other residue with a side group at the C-5 position of a monolignol ring, and said transformed plant having an untransformed natural wild-type plant whose lignin contains no syringyl residues, or corresponding other residues with a side group at the C-5 position of a monolignol ring.

29. A biomass according to claim 28, resulting from growing and harvesting a genetically transformed plant or plant precursor as defined in any one of claims 15 to 27.

30. A method of producing a cellulose-containing pulp useful for papermaking and the like, which comprises finely dividing a lignin-containing biomass derived from a gymnosperm plant to produce pulped mass containing lignin, and removing at least some of said lignin from said pulped mass, characterized in that said gymnosperm

plant is a genetically transformed plant producing lignin containing syringyl residues or other residues with a side group at the C-5 position of a monolignol ring.

31. A process of producing a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in production of at least one residue of a lignin biosynthetic pathway having a hydroxy group at the C-5 position of a monolignol ring, which process comprises:

providing a vector containing at least one expressible transgene that results in production of at least one residue having a hydroxy group at the C-5 position of a monolignol ring;

introducing said vector into cells of a gymnosperm to produce transformed cells;

regenerating transformed gymnosperm callus or shoots from the transformed cells;

maturing embryos from the transformed callus or shoots; and

generating transformed plant embryos, seeds, seedlings or plants from the matured embryos or shoots.

32. A process of producing a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene encoding an enzyme enabling hydroxylation at the C-5 position of a monolignol ring of at least one residue in a lignin biosynthetic pathway, which process comprises:
providing a vector containing at least one expressible transgene encoding an enzyme enabling hydroxylation at the C-5 position of a monolignol ring of at least one residue;

introducing said vector into cells of a gymnosperm to produce transformed cells;

regenerating transformed gymnosperm callus or shoots from the transformed cells;

maturing embryos from the transformed callus or shoots; and

generating transformed plant embryos, seeds, seedlings or plants from the matured embryos or shoots.

33. The process of claim 32, wherein the enzyme is selected from the group comprising ferulate-5-hydroxylase and homologs thereof.

34. A process of producing a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in hydroxylation of at least one residue of a lignin biosynthetic pathway at the C-5 position of a monolignol ring, which process comprises:

providing a vector containing at least one expressible transgene that results in hydroxylation of at least one residue at the C-5 position of a monolignol ring;

introducing said vector into cells of a gymnosperm to produce transformed cells;

regenerating transformed gymnosperm callus or shoots from the transformed cells;

maturing embryos from the transformed callus or shoots; and

generating transformed plant embryos, seeds, seedlings or plants from the matured embryos or shoots.

35. A transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in hydroxylation of at least one residue of a lignin biosynthetic pathway at the C-5 position of a monolignol ring.

36. A transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that encodes at least one enzyme enabling the production of a residue of a lignin biosynthetic pathway with a side group at the C-5 position of a monolignol ring, during the biosynthesis of lignin.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
KIRBY, EADES, GALE, BAKER & POTVIN
Attn. Gale, Edwin J.
Box 3432, Station D
OTTAWA, ONTARIO K1P 6N9
CANADA

Date of mailing *(day/month/year)* 19/07/2000	
Applicant's or agent's file reference 41193-PT	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/CA 00/ 00074	International filing date *(day/month/year)* 31/01/2000
Applicant SILVAGEN INC. et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mireille Claudepierre

Form PCT/ISA/220 (July 1998)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
41193-PT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00074	31/01/2000	01/02/1999
Applicant		
SILVAGEN INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.

   [ ] None of the figures.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

International Application No
PCT/CA 00/00074

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 A01H5/00 D21C3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NZ 328 434 A (UNIV BRITISH COLUMBIA SUBSTITU) 27 May 1998 (1998-05-27) the whole document ---	1,2, 8-10,15, 21-23
X	WO 98 03535 A (CHAPPLE CLINTON C S ;PURDUE RESEARCH FOUNDATION (US)) 29 January 1998 (1998-01-29) the whole document ---	1-30
X	WO 97 23599 A (DU PONT ;PURDUE RESEARCH FOUNDATION (US); CHAPPLE CLINT (US)) 3 July 1997 (1997-07-03) the whole document ---	1-30

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2000	19/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 11205 A (FLETCHER CHALLENGE FORESTS LIM ;GENESIS RESEARCH & DEV CORP LI (NZ) 19 March 1998 (1998-03-19) the whole document	1-30
X	BOUDET A M ET AL: "LIGNIN GENETIC ENGINEERING" MOLECULAR BREEDING,NL,KLUWER ACADEMIC PUBLISHERS, DORDRECHT, vol. 2, 1 January 1996 (1996-01-01), pages 25-39, XP002025844 ISSN: 1380-3743 page 29	1-30
X	MEYER KNUT ET AL: "Ferulate-5-hydroxylase from Arabidopsis thaliana defines a new family of cytochrome P450-dependent monooxygenases." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 93, no. 14, 1996, pages 6869-6874, XP002036466 1996 ISSN: 0027-8424 page 6873	1-30
P,X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; May 1999 (1999-05) PILATE G ET AL: "Toward the alteration of lignin metabolism in a conifer species: Overexpression of a poplar COMT sequence in transgenic hybrid larch plants." Database accession no. PREV199900356612 XP002141284 abstract & JOURNAL OF EXPERIMENTAL BOTANY, vol. 50, no. SUPPL., May 1999 (1999-05), page 31 Annual Meeting of the Society for Experimental Biology;Edinburgh, Scotland; March 22-26, 1999 ISSN: 0022-0957	1,2,15
P,X	WO 99 31243 A (CARRAWAY DANIEL T ;INT PAPER CO (US); CHIANG VINCENT L (US); SMELT) 24 June 1999 (1999-06-24) the whole document	1-30

-/--

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; AN 128:125902, WALTER, C., ET AL.: "Transformation and gene expression in Pinus radiata" XP002141285 abstract & FRI BULL. (1997), 203(IUFRO '97, GENETICS OF RADIATA PINE), 319-332,	1-30
A	US 5 681 730 A (ELLIS DAVID E) 28 October 1997 (1997-10-28) the whole document	1-30
A	WO 97 01641 A (NEW ZEALAND FOREST RESEARCH IN ;SMITH DALE RAYMOND (NZ); WALTER CH) 16 January 1997 (1997-01-16) the whole document	1-30
A	US 5 824 842 A (MACKAY JOHN ET AL) 20 October 1998 (1998-10-20) the whole document	1-30
A	BOUDET A -M: "LA LIGNIFICATION DOMESTIQUEE" BIOFUTUR,FR,BIOFUTUR, PARIS, vol. 158, 1 July 1996 (1996-07-01), pages 27-31, XP002065204 ISSN: 0294-3506 page 29	1-30

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
NZ 328434	A	27-05-1998	CA	2211665 A	24-01-1999
			US	5973228 A	26-10-1999
WO 9803535	A	29-01-1998	AU	716066 B	17-02-2000
			AU	3733297 A	10-02-1998
			CA	2260907 A	29-01-1998
			EP	0932612 A	04-08-1999
WO 9723599	A	03-07-1997	AU	716369 B	24-02-2000
			AU	1423997 A	17-07-1997
			BR	9612220 A	01-02-2000
			EP	0868432 A	07-10-1998
			NZ	325676 A	29-09-1999
			US	5981837 A	09-11-1999
WO 9811205	A	19-03-1998	US	5850020 A	15-12-1998
			AU	4403697 A	02-04-1998
			BR	9711745 A	18-01-2000
			EP	0929682 A	21-07-1999
			US	5952486 A	14-09-1999
			ZA	9710451 A	20-05-1999
WO 9931243	A	24-06-1999	NONE		
US 5681730	A	28-10-1997	CA	2075135 A	03-02-1993
WO 9701641	A	16-01-1997	AU	6141796 A	30-01-1997
			CA	2161391 A	27-12-1996
US 5824842	A	20-10-1998	NONE		

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

GALE, Edwin, J.
Kibry, Eades, Gale, Baker
P.O. Box 3432, Station D
Ottawa, Ontario K1P 6N9
CANADA

Date of mailing (day/month/year)
19 October 2000 (19.10.00)

Applicant's or agent's file reference
41193-PT

IMPORTANT NOTIFICATION

International application No.
PCT/CA00/00074

International filing date (day/month/year)
31 January 2000 (31.01.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

SILVAGEN INC.
BC Research and Innovation Complex
3650 Wesbrook Mall
Vancouver, British Columbia V6S 2L2
Canada

State of Nationality: CA
State of Residence: CA
Telephone No.: (604)221-9893
Facsimile No.: (604)224-0540
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address

CELLFOR INC.
BC Research and Innovation Complex
3650 Wesbrook Mall
Vancouver, British Columbia V6S 2L2
Canada

State of Nationality: CA
State of Residence: CA
Telephone No.: (604)221-9893
Facsimile No.: (604)224-0540
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

A. Karkachi

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

GALE, Edwin, J.
Kibry, Eades, Gale, Baker
P.O. Box 3432, Station D
Ottawa, Ontario K1P 6N9
CANADA

Date of mailing (day/month/year) 19 October 2000 (19.10.00)	
Applicant's or agent's file reference 41193-PT	**IMPORTANT NOTIFICATION**
International application No. PCT/CA00/00074	**International filing date** (day/month/year) 31 January 2000 (31.01.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
SILVAGEN INC. BC Research and Innovation Complex 3650 Wesbrook Mall Vancouver, British Columbia V6S 2L2 Canada	CA	CA
	Telephone No. (604)221-9893	
	Facsimile No. (604)224-0540	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
CELLFOR INC. BC Research and Innovation Complex 3650 Wesbrook Mall Vancouver, British Columbia V6S 2L2 Canada	CA	CA
	Telephone No. (604)221-9893	
	Facsimile No. (604)224-0540	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin d s Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer A. Karkachi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

003595908

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
GALE, Edwin J.
KIRBY, EADES, GALE, BAKER
Box 3432, Station D
Ottawa, Ontario, K1P 6N9
CANADA

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*	18. 09. 00
Applicant's or agent's file reference: 41193-PT	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/ CA 00/ 00074	31/01/2000	01/02/1999

Applicant: CELLFOR INC. et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

30/08/2000

2. This date of receipt is:

- [X] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

[ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	WERNER N Tel. (+49-89) 2399-2635

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

GALE, Edwin J.
KIRBY, EADES, GALE, BAKER
Box 3432, Station D
Ottawa, Ontario, K1P 6N9
CANADA

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	22.11.2000
Applicant's or agent's file reference: 41193-PT	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00074	31/01/2000	01/02/1999

International Patent Classification (IPC) or both national classification and IPC

C12N15/82

Applicant

CELLFOR INC. et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☒	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☒	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 01/06/2001.

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner

Roscoe, R

Formalities officer (incl. extension of time limits)
Emslander, S
Telephone No. +49 89 2399 8718

ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

GALE, Edwin, J.
Kibry, Eades, Gale, Baker
P.O. Box 3432, Station D
Ottawa, Ontario K1P 6N9
CANADA

Date of mailing (day/month/year) 16 May 2000 (16.05.00)	
Applicant's or agent's file reference 41193-PT	**IMPORTANT NOTIFICATION**
International application No. PCT/CA00/00074	International filing date (day/month/year) 31 January 2000 (31.01.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 01 February 1999 (01.02.99)
Applicant SILVAGEN INC. et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
01 Febr 1999 (01.02.99)	60/118,124	US	09 May 2000 (09.05.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Sean Taylor

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 19 October 2000 (19.10.00)	
International application No. PCT/CA00/00074	**Applicant's or agent's file reference** 41193-PT
International filing date (day/month/year) 31 January 2000 (31.01.00)	**Priority date** (day/month/year) 01 February 1999 (01.02.99)
Applicant ELLIS, David, Dunham et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

30 August 2000 (30.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer A. Karkachi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00074

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 A01H5/00 D21C3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NZ 328 434 A (UNIV BRITISH COLUMBIA SUBSTITU) 27 May 1998 (1998-05-27) the whole document ---	1,2, 8-10,15, 21-23
X	WO 98 03535 A (CHAPPLE CLINTON C S ;PURDUE RESEARCH FOUNDATION (US)) 29 January 1998 (1998-01-29) the whole document ---	1-30
X	WO 97 23599 A (DU PONT ;PURDUE RESEARCH FOUNDATION (US); CHAPPLE CLINT (US)) 3 July 1997 (1997-07-03) the whole document ---	1-30
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2000	19/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Maddox, A

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 11205 A (FLETCHER CHALLENGE FORESTS LIM ;GENESIS RESEARCH & DEV CORP LI (NZ) 19 March 1998 (1998-03-19) the whole document	1-30
X	BOUDET A M ET AL: "LIGNIN GENETIC ENGINEERING" MOLECULAR BREEDING,NL,KLUWER ACADEMIC PUBLISHERS, DORDRECHT, vol. 2, 1 January 1996 (1996-01-01), pages 25-39, XP002025844 ISSN: 1380-3743 page 29	1-30
X	MEYER KNUT ET AL: "Ferulate-5-hydroxylase from Arabidopsis thaliana defines a new family of cytochrome P450-dependent monooxygenases." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 93, no. 14, 1996, pages 6869-6874, XP002036466 1996 ISSN: 0027-8424 page 6873	1-30
P,X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; May 1999 (1999-05) PILATE G ET AL: "Toward the alteration of lignin metabolism in a conifer species: Overexpression of a poplar COMT sequence in transgenic hybrid larch plants." Database accession no. PREV199900356612 XP002141284 abstract & JOURNAL OF EXPERIMENTAL BOTANY, vol. 50, no. SUPPL., May 1999 (1999-05), page 31 Annual Meeting of the Society for Experimental Biology;Edinburgh, Scotland; March 22-26, 1999 ISSN: 0022-0957	1,2,15
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; AN 128:125902, WALTER, C., ET AL.: "Transformation and gene expression in Pinus radiata" XP002141285 abstract & FRI BULL. (1997), 203(IUFRO '97, GENETICS OF RADIATA PINE), 319-332,	1-30
A	US 5 681 730 A (ELLIS DAVID E) 28 October 1997 (1997-10-28) the whole document	1-30
A	WO 97 01641 A (NEW ZEALAND FOREST RESEARCH IN ;SMITH DALE RAYMOND (NZ); WALTER CH) 16 January 1997 (1997-01-16) the whole document	1-30
A	US 5 824 842 A (MACKAY JOHN ET AL) 20 October 1998 (1998-10-20) the whole document	1-30
A	BOUDET A -M: "LA LIGNIFICATION DOMESTIQUEE" BIOFUTUR,FR,BIOFUTUR, PARIS, vol. 158, 1 July 1996 (1996-07-01), pages 27-31, XP002065204 ISSN: 0294-3506 page 29	1-30

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/00074

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			CA	2161391 A	27-12-1996
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G.S.T. No
R121887095

CELLFOR – 41193-PT – **NO FEE**

March 15, 2001

VIA FACSIMILE
(Original by Registered Mail)

IPEA
European Patent Office
D-280298 Munich
Germany

[redacted]

Dear Sirs:

Re:

International Application No.:	PCT/CA00/00074
International Filing Date:	January 31, 2000
Applicant:	Cellfor Inc. et al
Inventor:	ELLIS, David Dunham et al.

This is with reference to the Written Opinion of November 22, 2000. An extension of time to reply to the Written Opinion was granted on March 5, 2001 so that the new deadline for response is ***March 22, 2001***.

IN THE CLAIMS

Please replace pages 19-24 & 24/1 containing claims 1-36 by new pages 19-25 containing claims 1-36, one copy of which is enclosed.

REMARKS

The Applicant provides the following comments using the same headings as those found in the Written Opinion.

KIRBY EADES GALE BAKER

I. Basis

The Examiner refused to examine claims 31 - 36 since they are considered to contain subject-matter extending beyond that disclosed in the application as originally filed. These claims refer to hydroxy residues at the C-5 position of the monolignol ring and enzymes enabling hydroxylation at this position. The Examiner maintained that this cannot be found in the application as originally filed.

However, when the F5H gene is introduced into a tree genome, the only modification that can take place is the hydroxylation of the C-5 carbon. After all, F5H stands for ferrulate 5-hyroxylase. It should also be noted that in Figure 1, final line, a metabolic route is shown from coniferyl alcohol to sinapyl alcohol via transformations with F5H and OMT (O-methyl transferase). The F5H adds a hydroxy moiety to the 5-carbon and then the OMT methylates the hydroxy. However, between these two steps, there is reference to a novel mono-lignol, which would clearly have hydroxy at the 5-position since it is not yet methylated by the OMT.

It is therefore believed that claims 31 - 36 are supported by the original application and should be examined.

II. Priority

The Examiner considered that any subject matter relating to "other residues with a side group at the C-5 position of the monolignol ring" lacks support in the priority document, so that claims including such a feature cannot claim priority from that document.

It should be noted, however, that the object of the invention stated in the priority document on page 5 at lines 15 to 18 is to provide modified monolignol residues, and preferably, a greater content of syringyl residues. Thus, the application clearly contemplates residues other than just syringyl.

The Examiner's attention is also directed to page 10, line 28 to page 11, line 3 where there is a very broad statement regarding the nature of the invention, namely that the invention includes the use of any genes that make the plants more commercially desirable. This would include genes that insert other residues at the C-5 position. All aspects of the present invention are covered in a general sense by such general statements in the priority document.

Consequently, the priority document is entirely consistent with all of the claimed subject matter and the claims should therefore be regarded as fully supported by the priority document.

III. No Opinion

In view of the comments above in section I, it is believed that Applicant is entitled to an opinion on claims 31-36.

KIRBY EADES GALE BAKER

IV. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

The Examiner is of the opinion that D1 anticipates claims 1-4, 8-12, 15-17, 22, 24, 25 and 28-30.

Applicant believes that the Examiner is incorrect in that D1 has no resemblance to the present application. Beta-glucosidase (CBG) catalyses a reaction which removes a glucose residue from the monolignol prior to polymerization into lignin. It has absolutely nothing to do with the type of lignin made, the form of the monolignol or the relative ratio of S & G units. There are many things that can alter the general levels of lignins in the plants and in contrast the present application covers changes in lignin composition not absolute levels of lignin. Therefore, this patent does not support the Examiners opinion on lack of novelty.

References D2 and D3 speculate that it is possible that F5H could be the gene that is missing in gymnosperms which account for the absence of syringyl lignin; however, this is simply speculation. Neither of the references use this gene in a gymnopserm nor do they show that this can be done in gymnosperms. Prior to this, no one had modified a gymnosperm for altered lignin, hence it is impossible for anyone to say that this is obvious and not novel. In fact, the use of this gene to enact the hydroxylation of the C-5 position in gymnosperm would not be obvious to someone skilled in the art based on these references.

The further assertion that the transformation of conifers is routine is flawed. There are less than a dozen labs in the world who can reliably transform conifers most of these work with only one or two genotypes. Conifer transformation can be done, but it is not routine. Applicant would further suggest that just because conifer transformation can be done, it does not make any difference to the novelty of the rejected claims as there is not prior teaching, only far fetched speculation that this gene may account for the difference between gymnosperms and angiosperms.

In the case of references D4 and D5, there is no question that one can modify lignin and the relative ratio of syringyl units in angiosperms with the over-expression of F5H. However, these angiosperm systems all had pre-existing syringyl pathways. Thus modification involved only changing the relative flow through an already existing pathway. This is not the case in the present application as no pre-existing pathway existed for the hydroxylation of the C-5 carbon of a monlignol. Hence, Applicant has created a new, novel pathway that never previously existed. Therefore, the simple fact that a person can modify an existing pathway does not remove the novelty or make the engineering of a new unique pathway that never preciously existed obvious. In fact it does not add anything to the argument as it is comparing apples and oranges.

Inventive step

For the reasons given above, all of the claims are believed to be inventive as well as novel.

KIRBY EADES GALE BAKER

V. Certain Documents

The Examiner drew Applicant's attention to D8. However, no comments are provided regarding this document as it was published after the priority date of the present application.

VI. Certain Observations

In view of the Examiner's objection to the wording of claim 2, this claim has been amended to correspond to the description on page 11 at lines 16 and 17.

Regarding the Examiner's objection to claim 5 and claim 18, the expression "substantially homologous activity" has been changed to "substantially equivalent function". This terminology is based on the description on page 7, line 4.

Regarding the Examiner's objection to claims 6, 7, 19 and 20 concerning the term "homology", it is believed that the objection is unfounded. The Cambridge Dictionary of Biology, edited by Peter Walker, 1990, defines "homology" in the following terms:

(1) Morphological equivalence, common evolutionary origin.
(2) Refers to the degree of similarity between of DNA or peptide sequences.

It is definition (2) that is clearly intended in the claims objected to, and thus the claims are believed to be quite clear in meaning.

Regarding claim 28, the wording of the claim has been modified to refer to an untransformed plant relative for logical accuracy.

Yours very truly,

Original Signed By
EDWIN J. GALE

Edwin Gale
EG/fb
Encl.

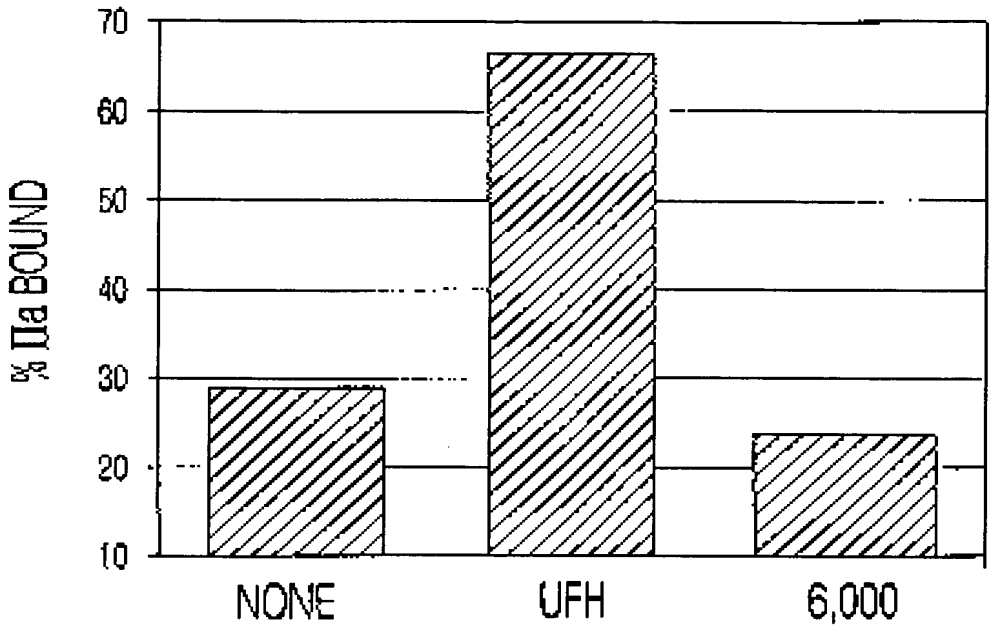


Fig. 9

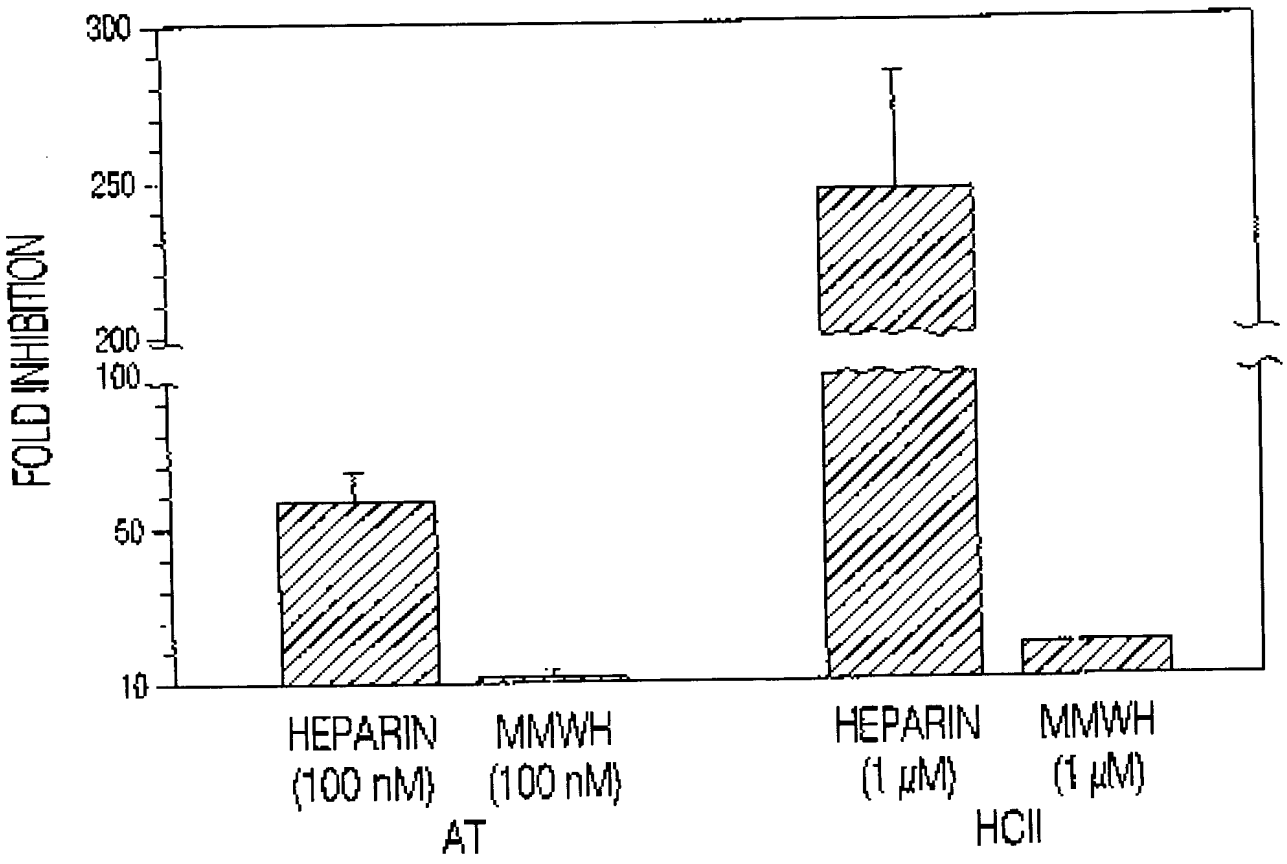


Fig. 10

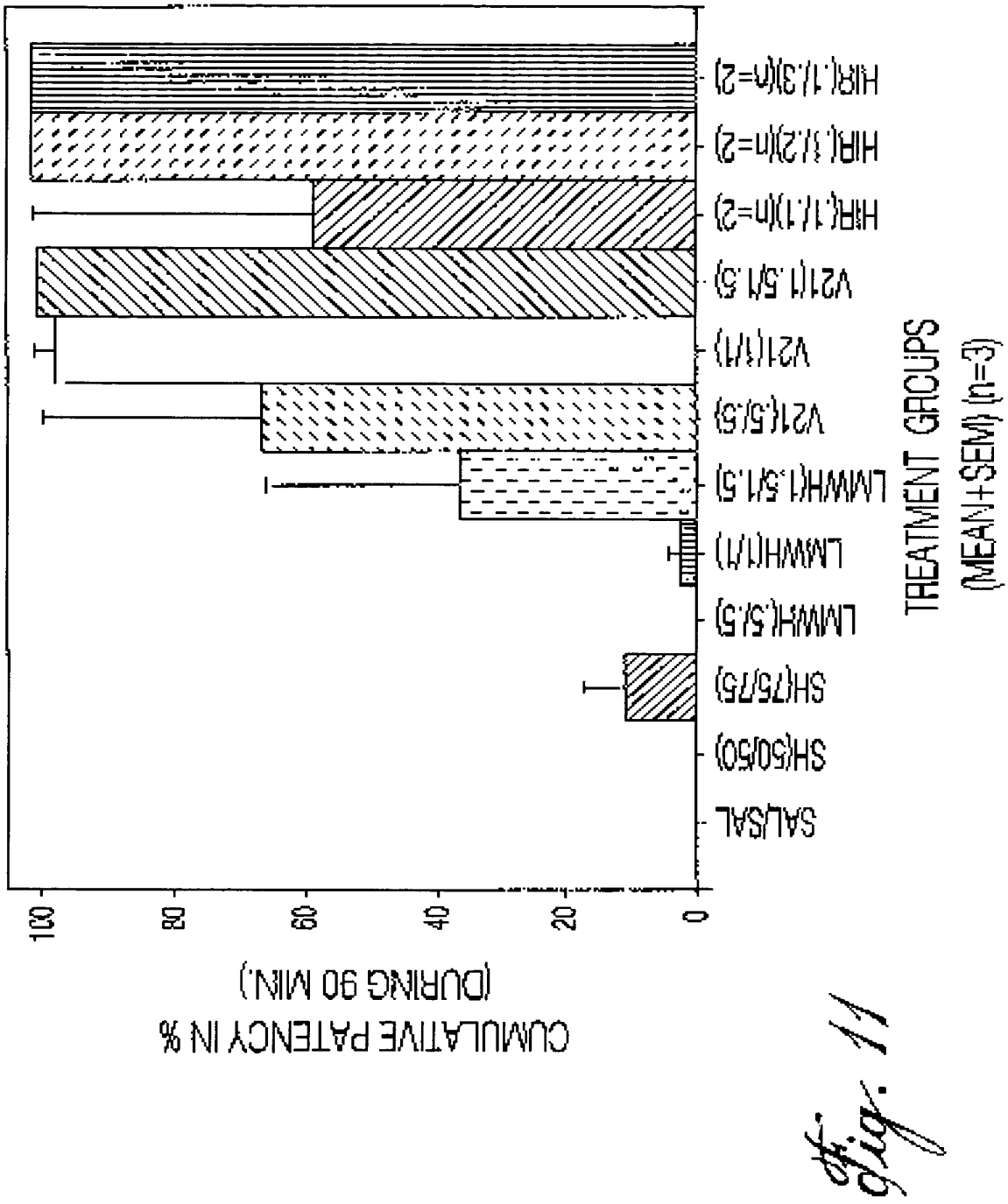


Fig. 11

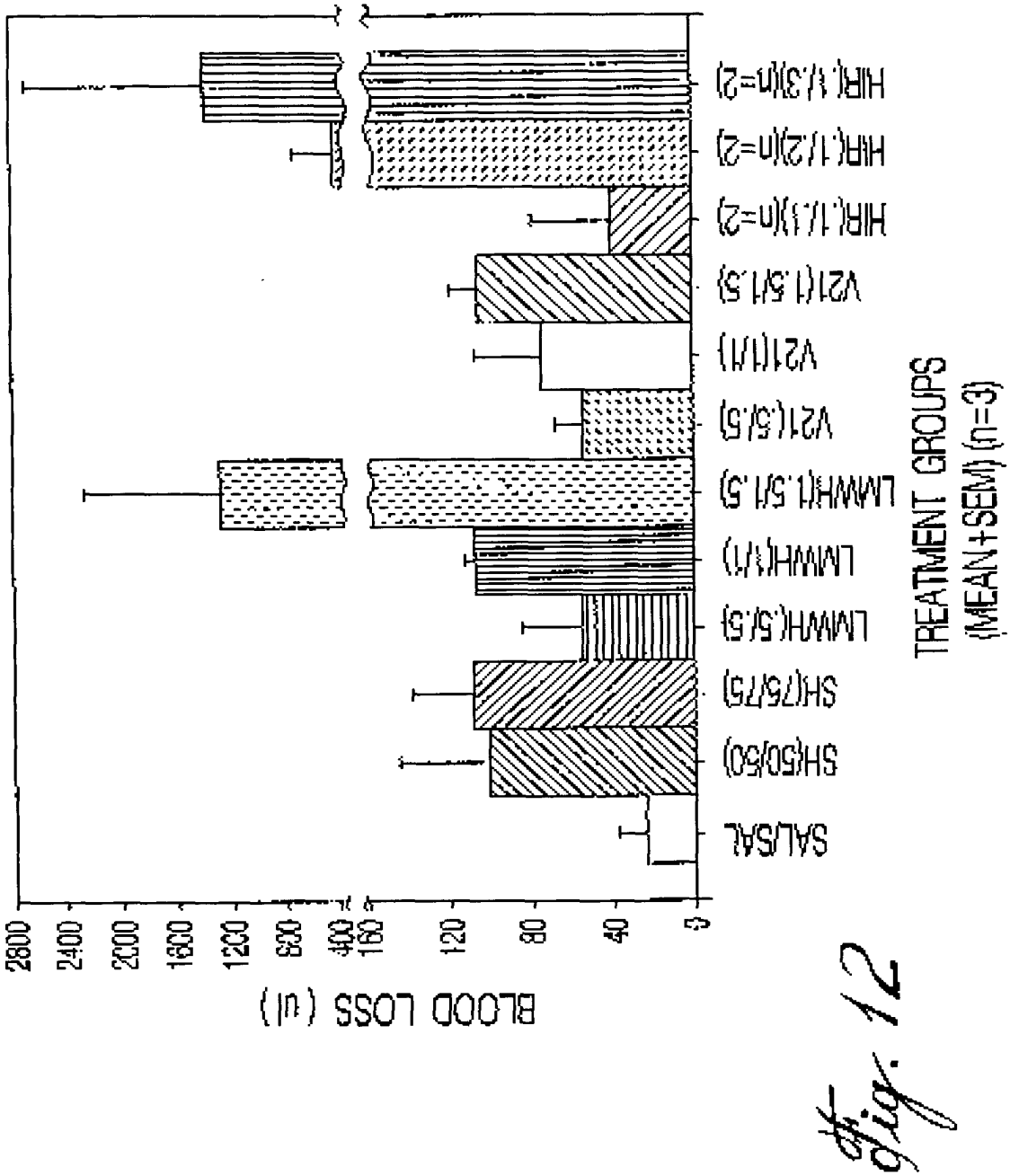
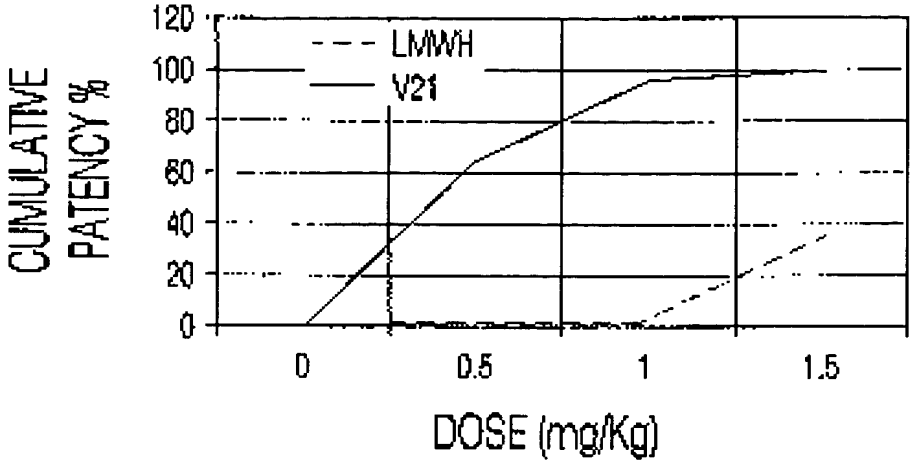


Fig. 12



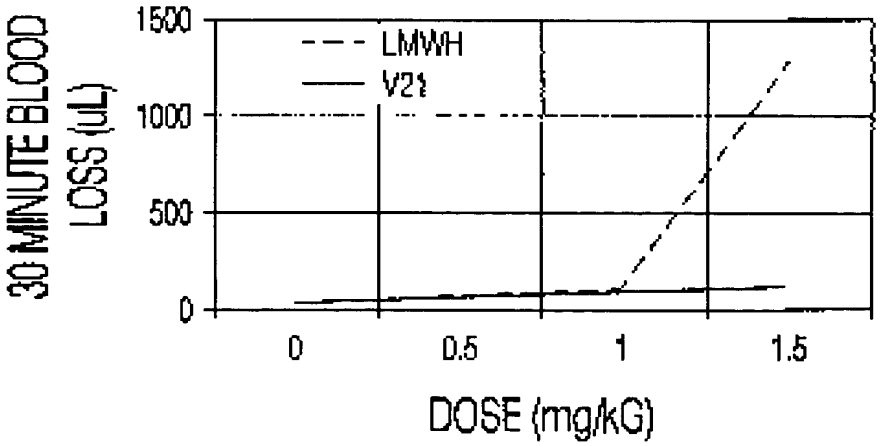


Fig. 13

	LMWH	V21		
0	21	21	0	0
0.5	24.7	23.1	12.4	1.12
1	24.3	30.7	2.37	f0.1
1.5	39.4	52.1	2.98	2.31

Comparative effects of V21 and LMWH on APTT

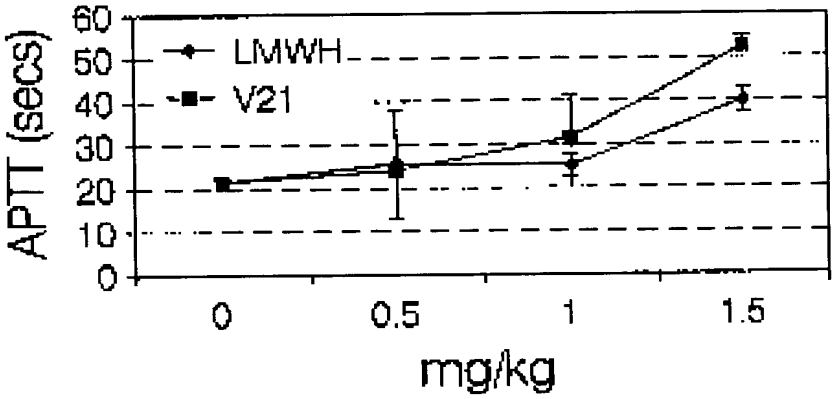


Fig. 14

LMWH	V21	lmwhse	V21 se	
0	0	0	0	0
0.5	1.1	0.8	0.19	0.32
1	2.8	1.5	0.91	0.32
1.5	2.6	2.8	0.06	0.36

Comparative effects of LMWH and V21 on the anti-Xa level

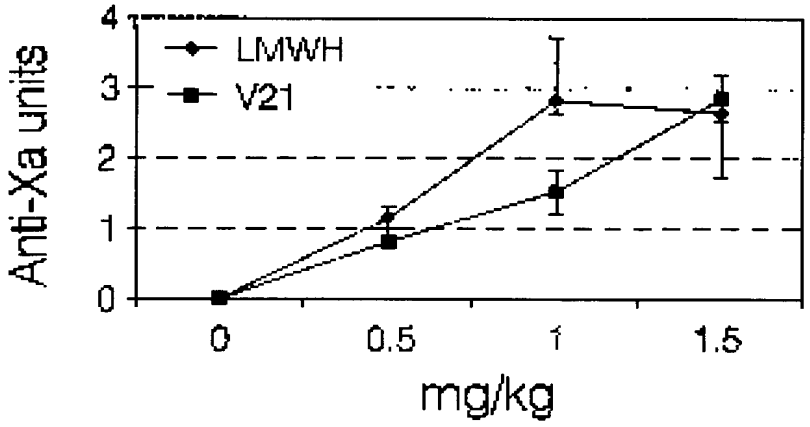


Fig. 15

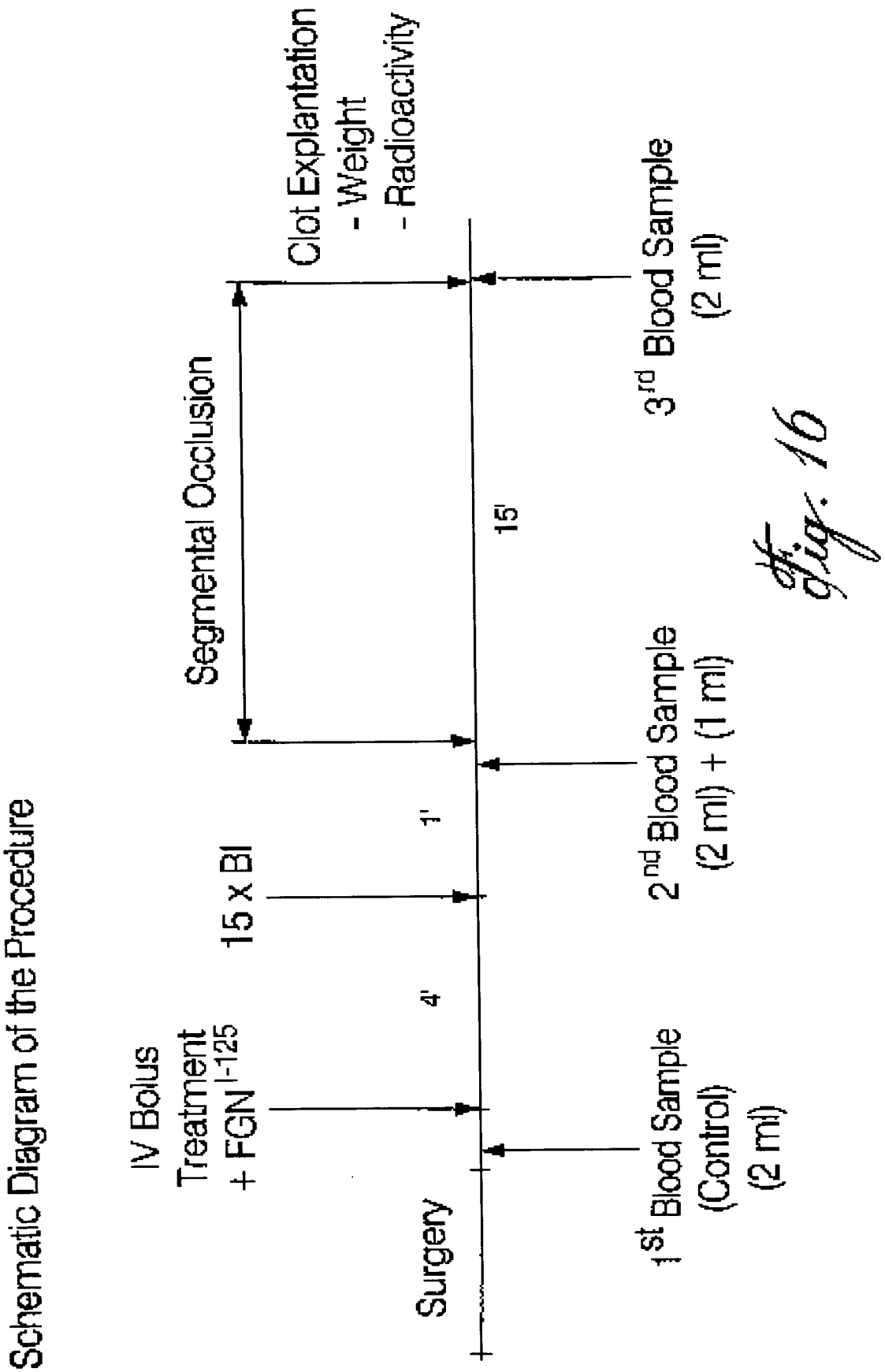


Fig. 16

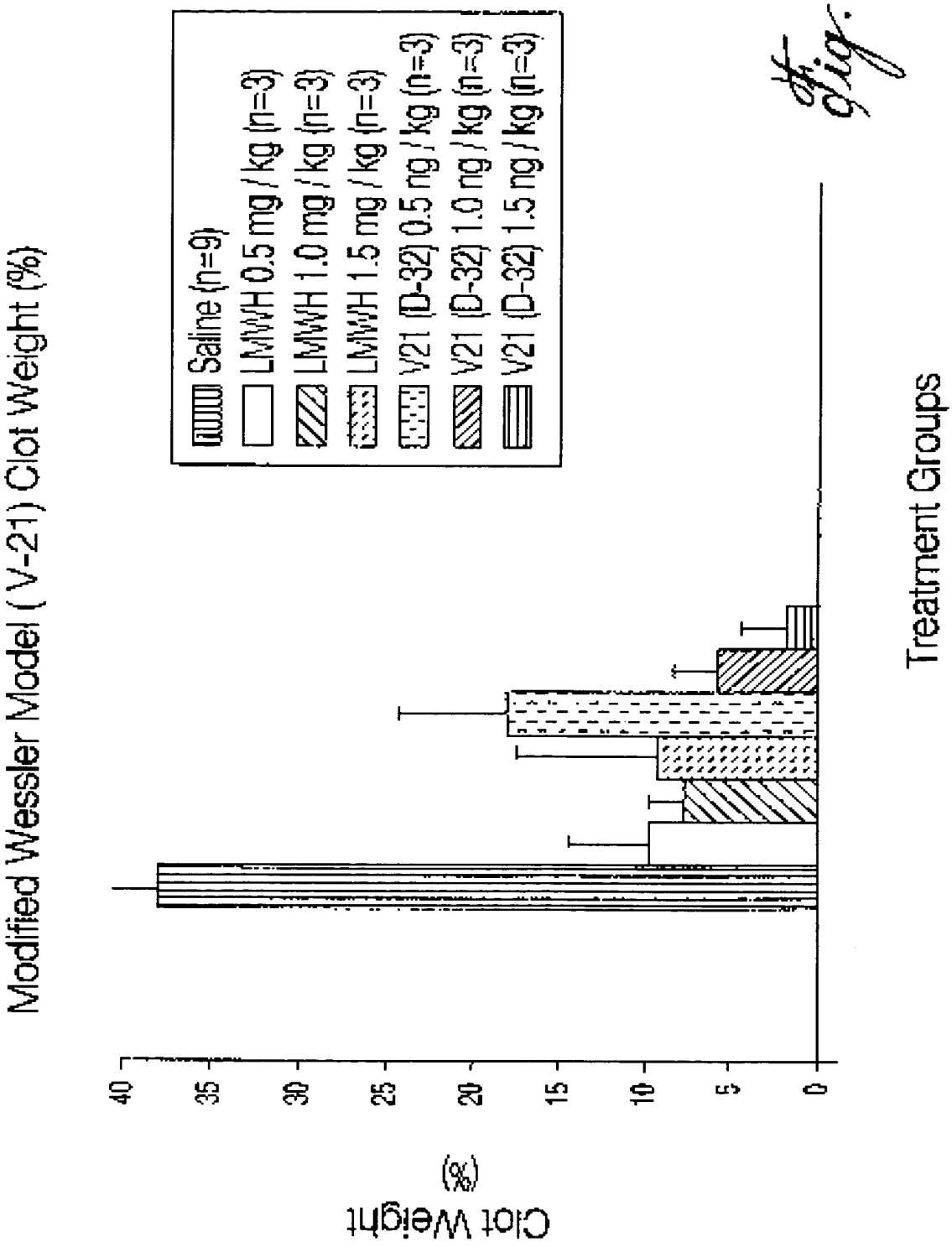


Fig. 17

	LMWH	V21		
0	37.8	37.8	3.7	3.7
0.5	9.6	18	4.7	6.1
1	7.5	5.6	2.1	2.6
1.5	9.1	1.7	8.2	0.6

Comparison of LMWH and V21:Prophylaxis model

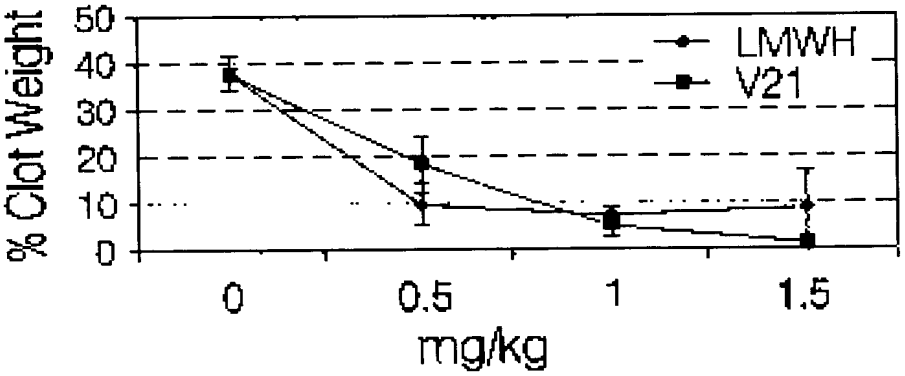


Fig. 18

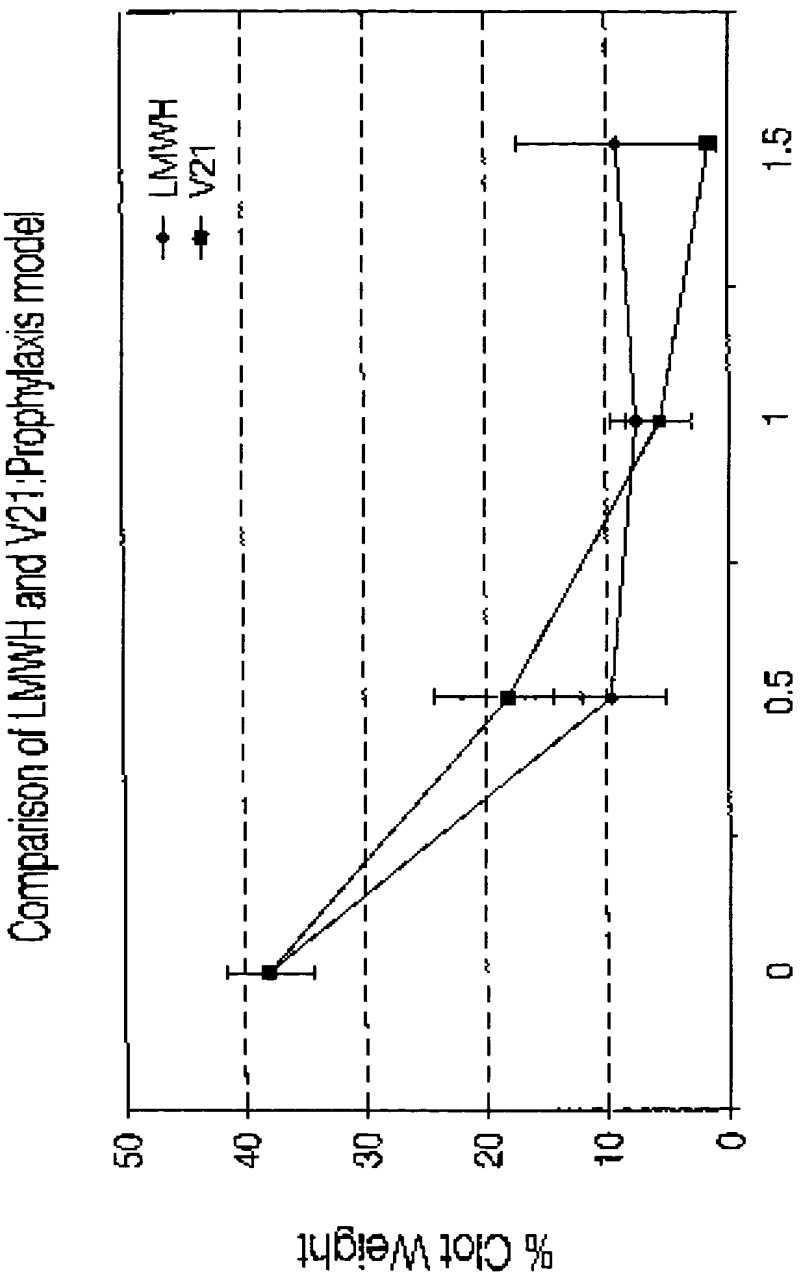


Fig. 19

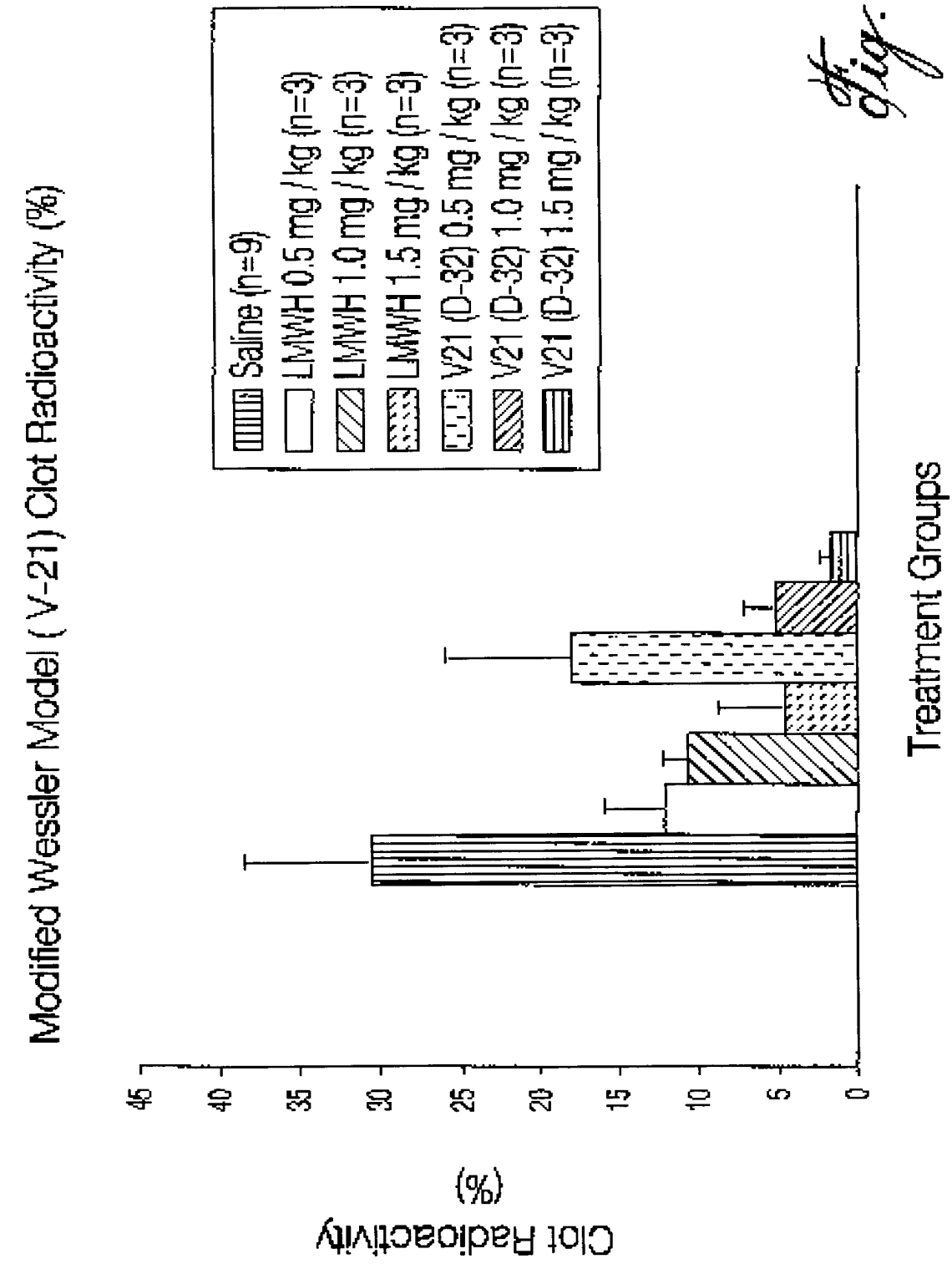


Fig. 20

	LMWH	V21	se	se
0	30	30	8	8
0.5	12	18	3.8	7.8
1	11	5	1.7	2.1
1.5	4.5	1.6	4.2	0.7

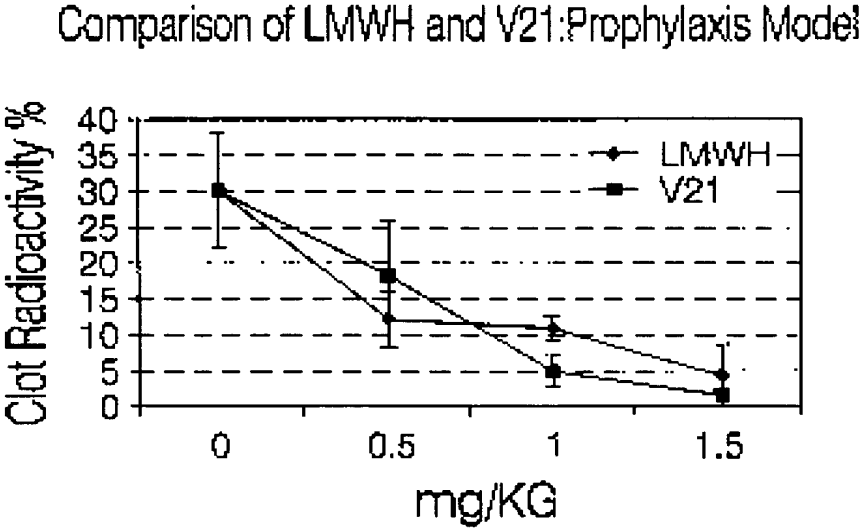


Fig. 21

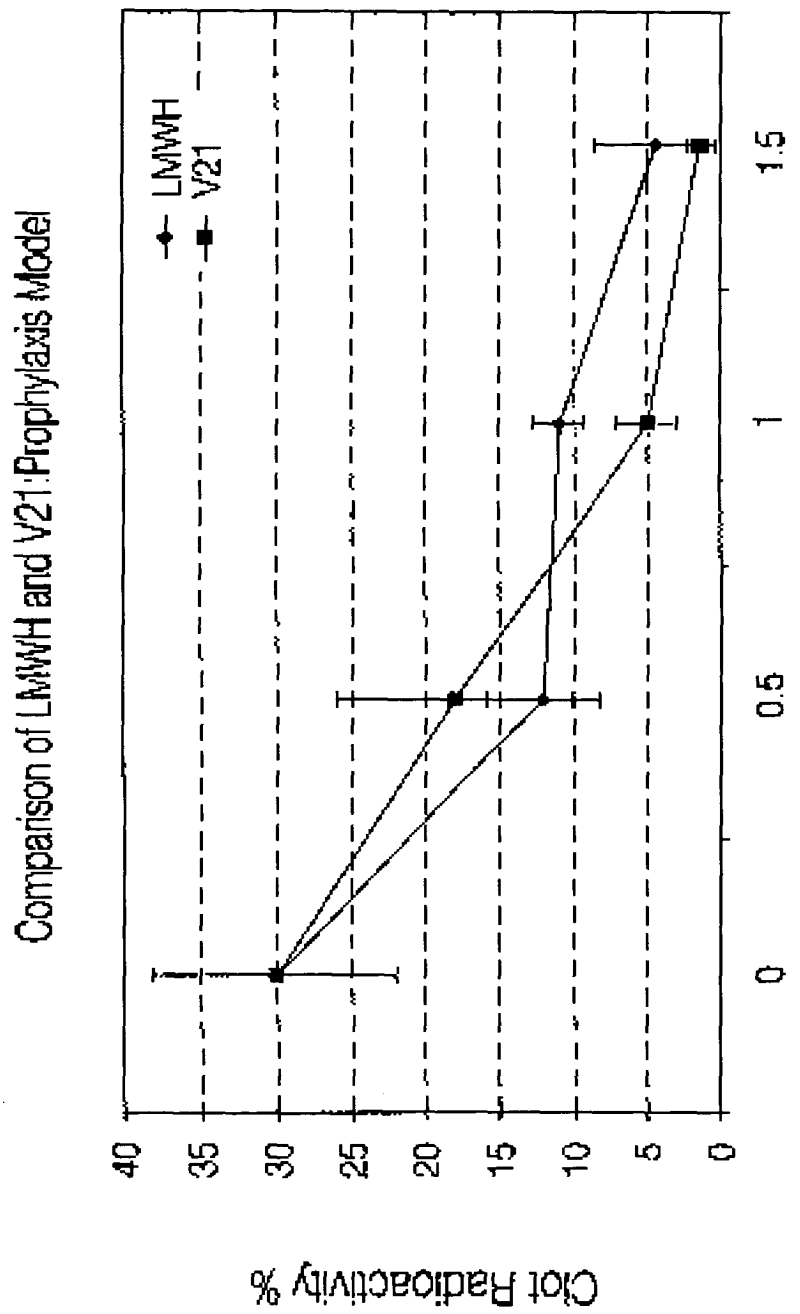


Fig. 22

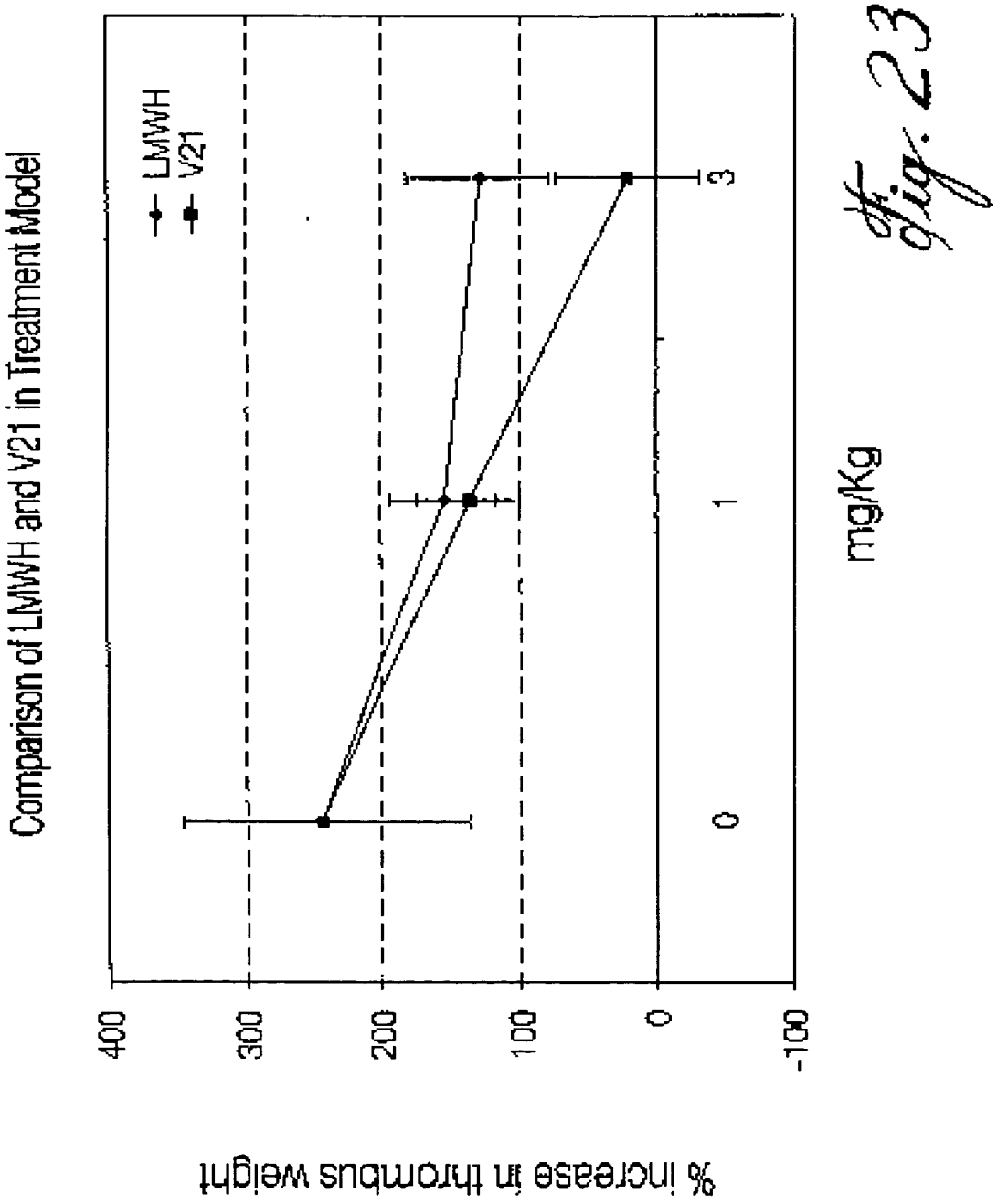


Fig. 23

	LMWH	V21		
0	242	242	104	104
1	155	137	37	30
3	129	21	52	31

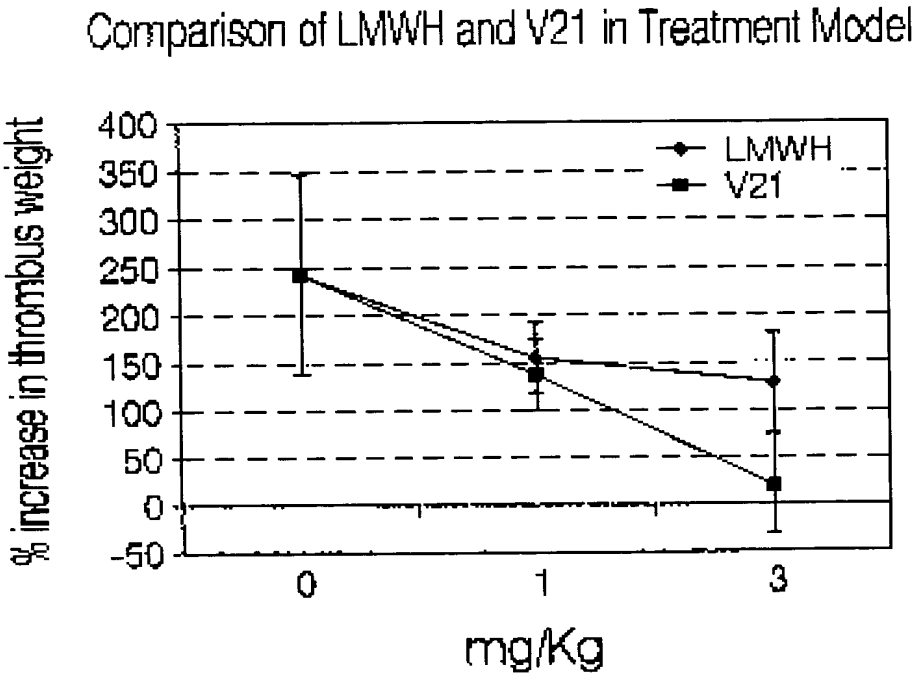


Fig. 24

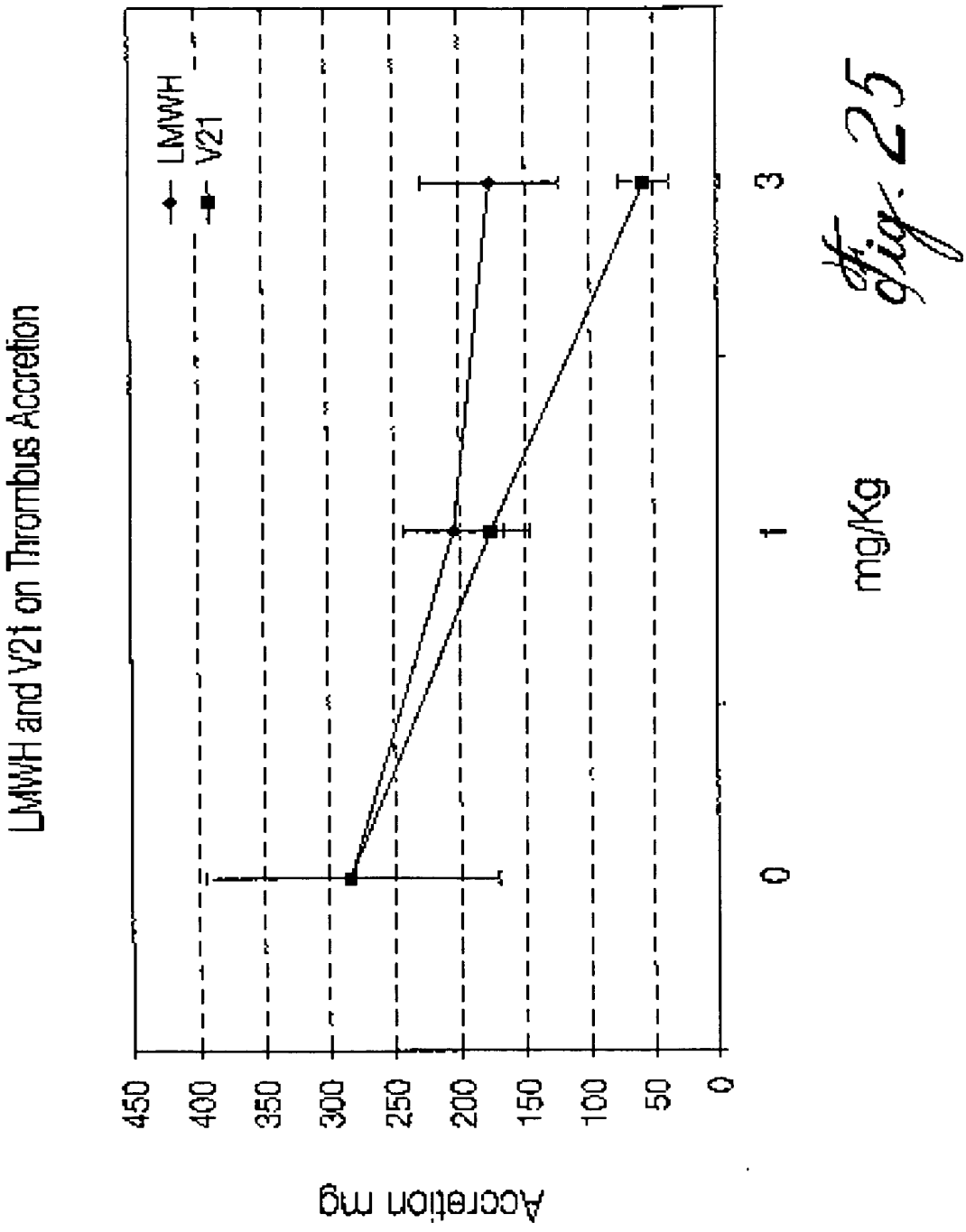


Fig. 25

	LMWH	V21		
0	282	282	111	111
1	202	174	37	29
3	174	57	51	20

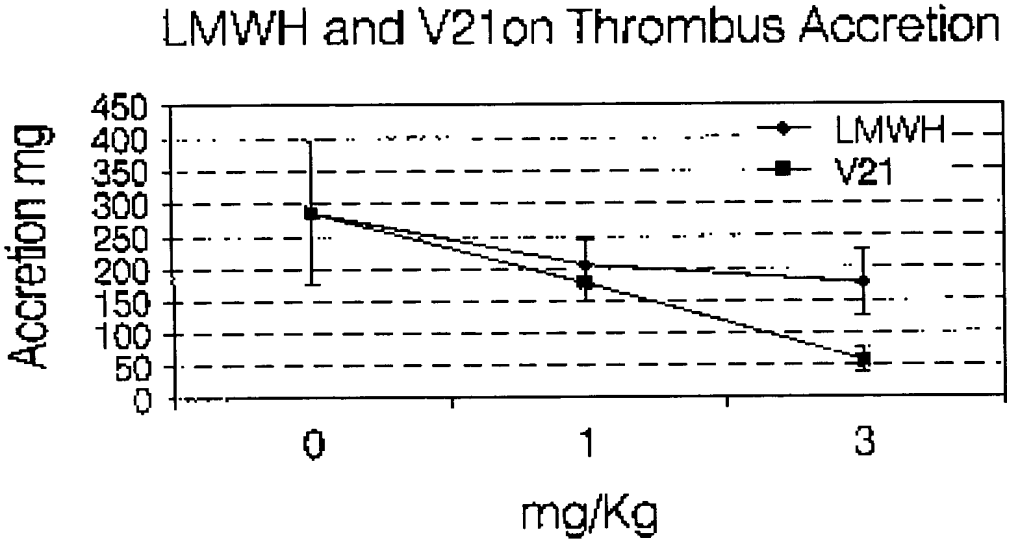


Fig. 26

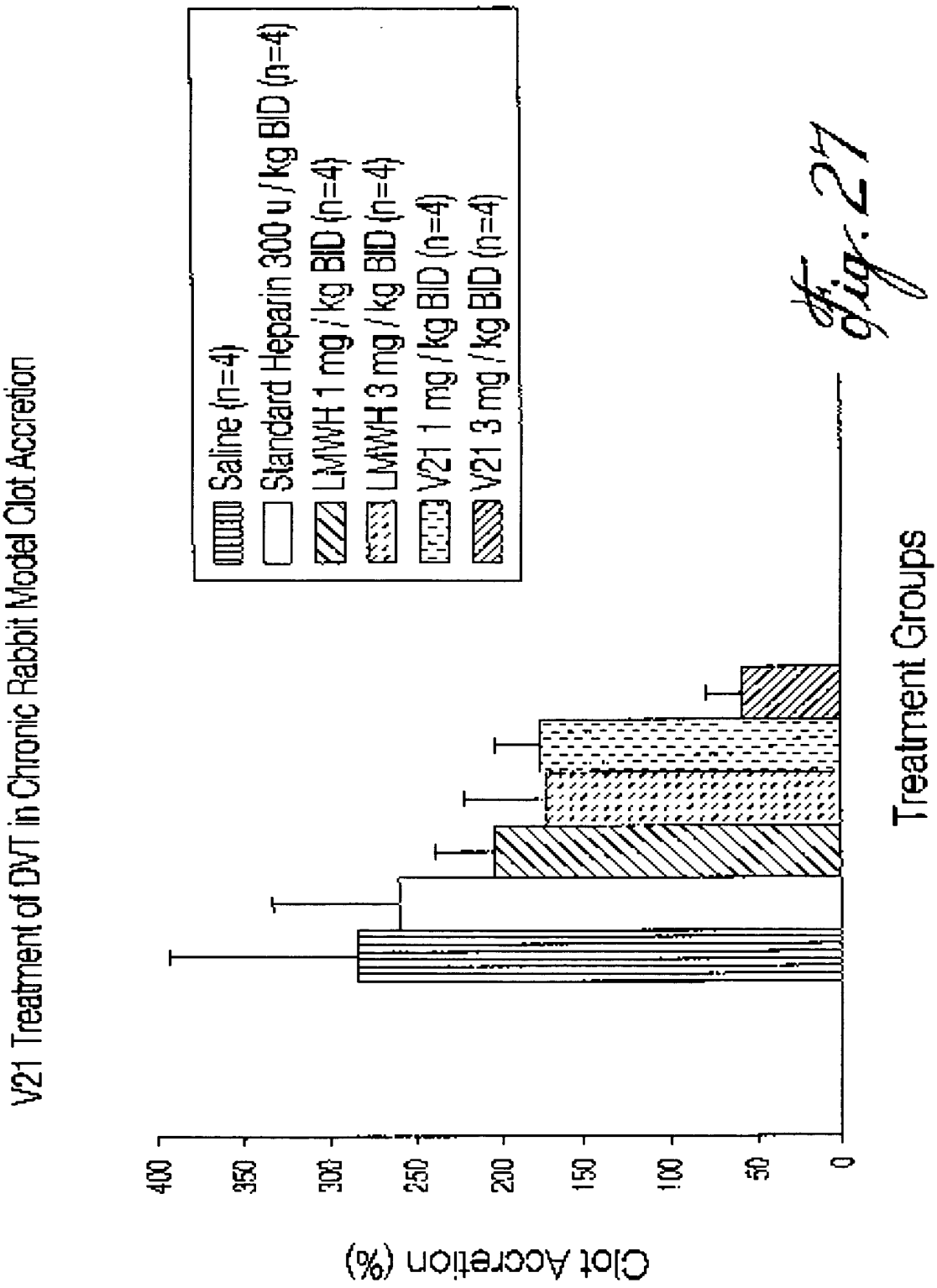


Fig. 21

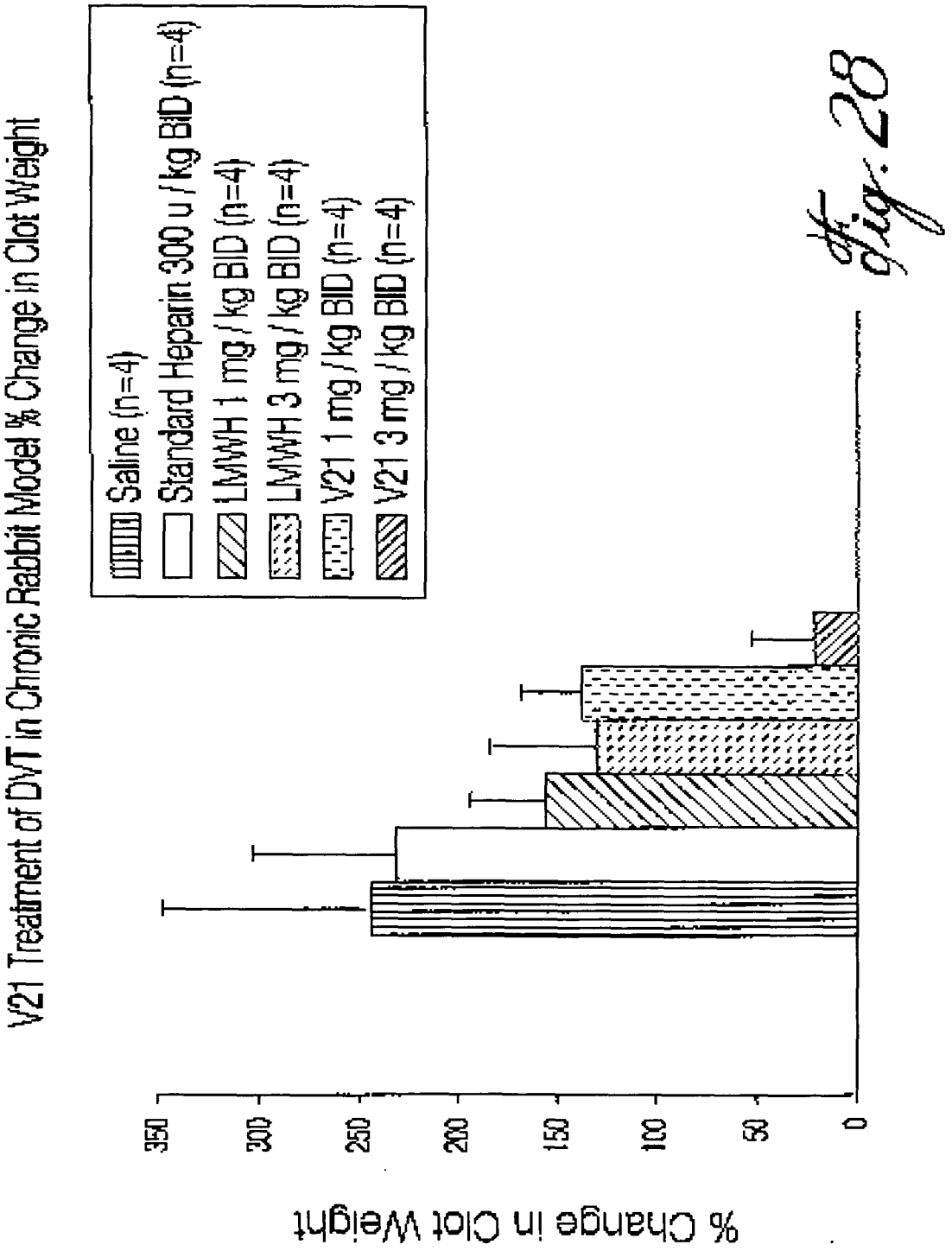


Fig. 28

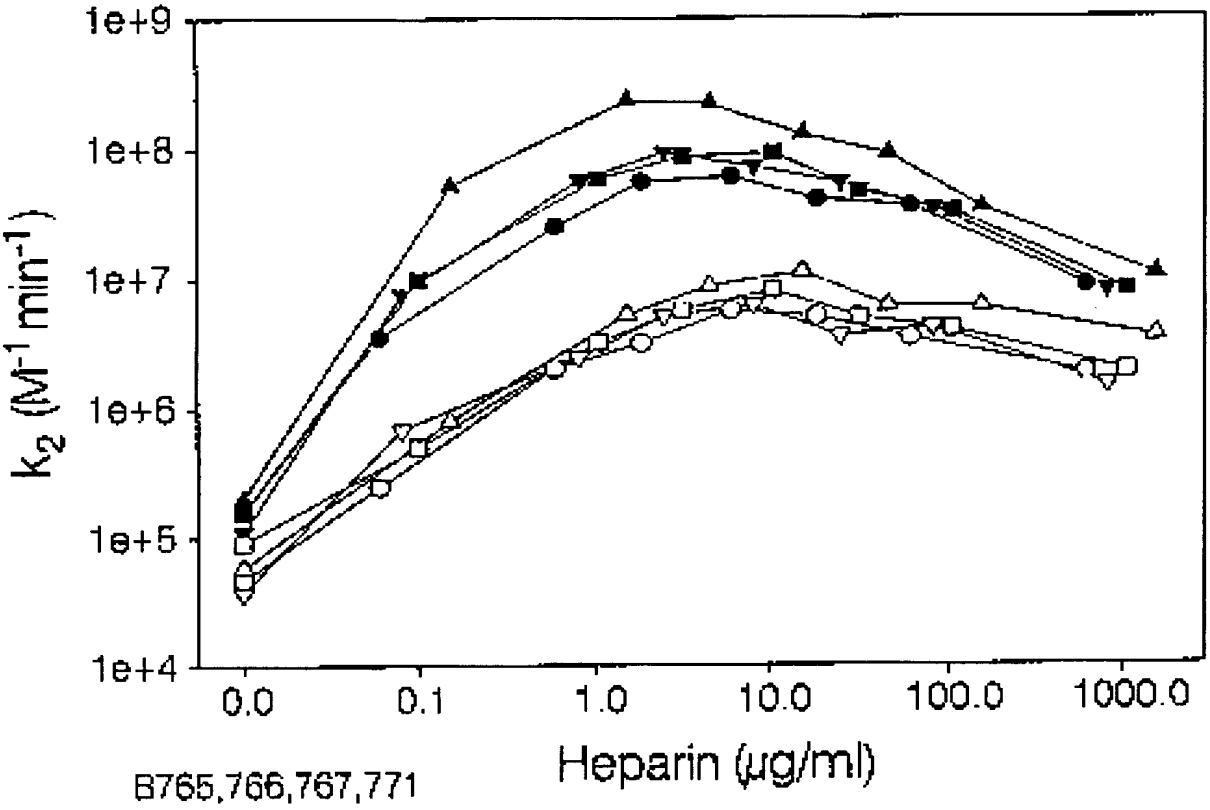


Fig. 29

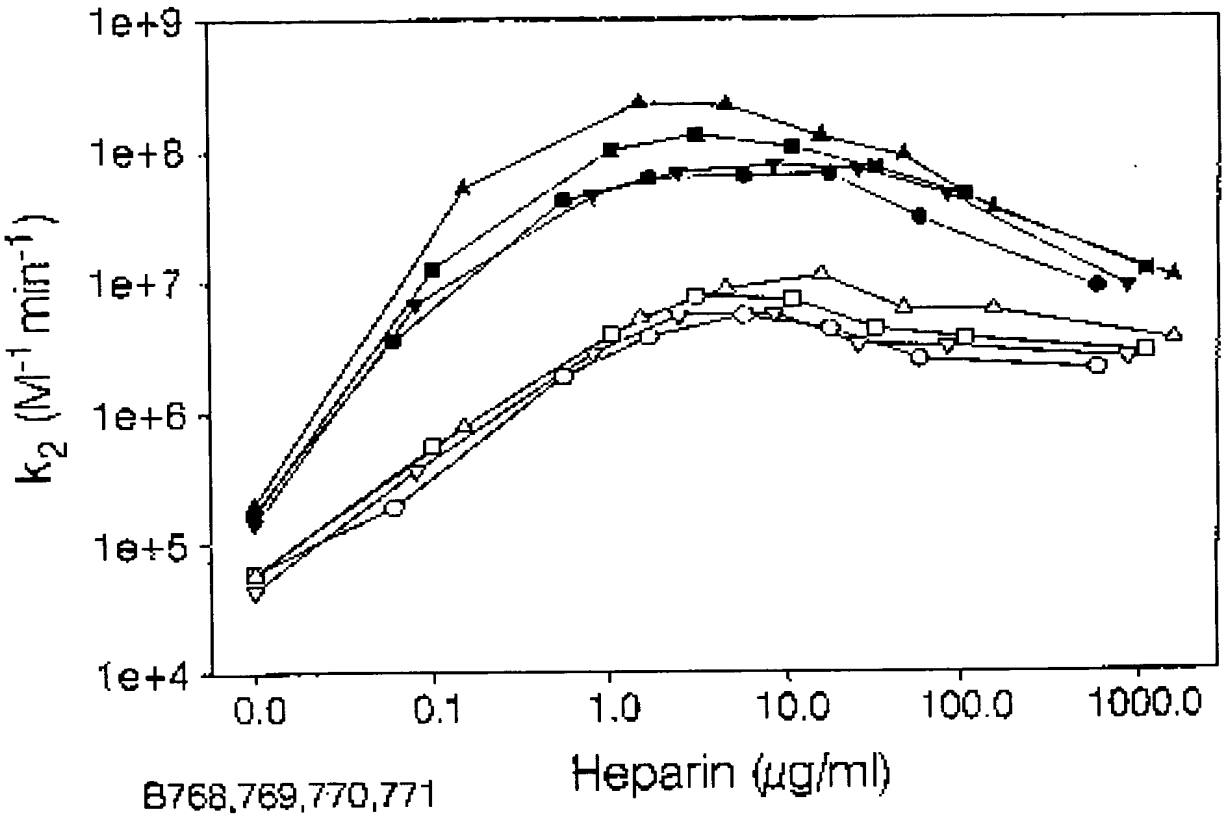


Fig. 30

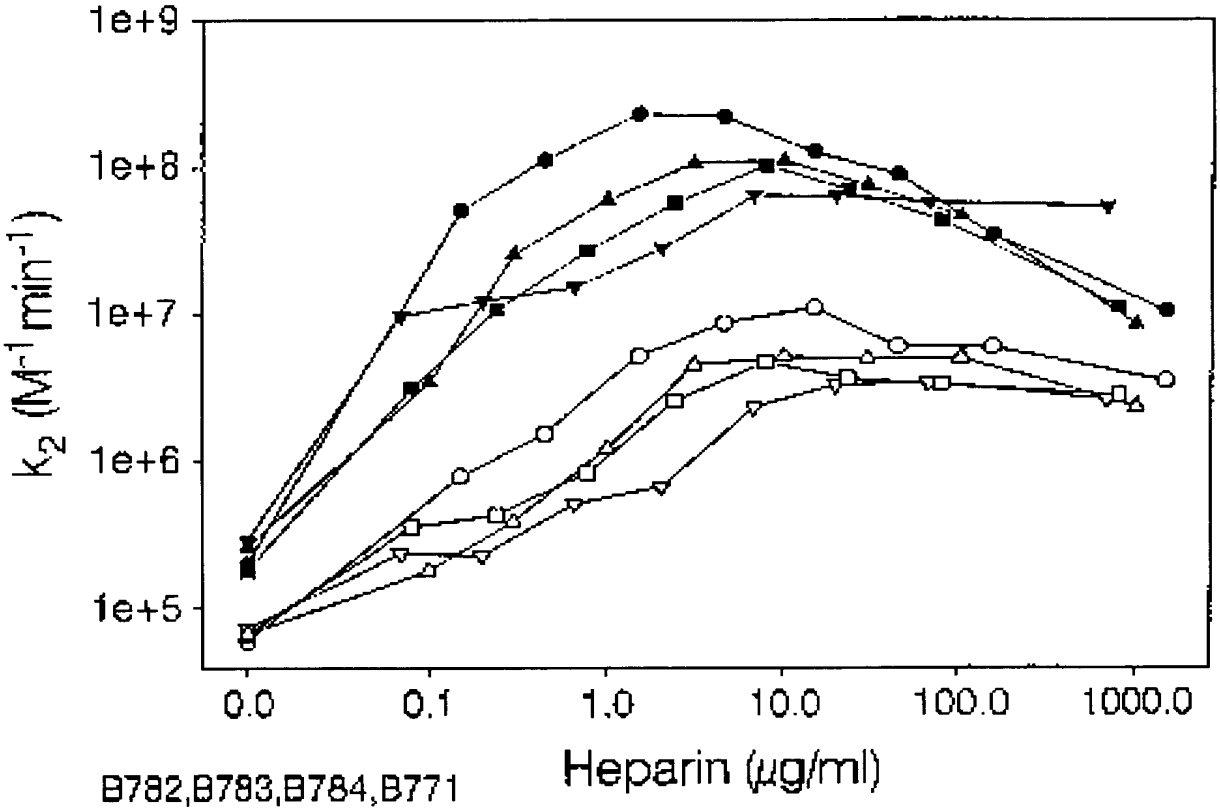


Fig. 31

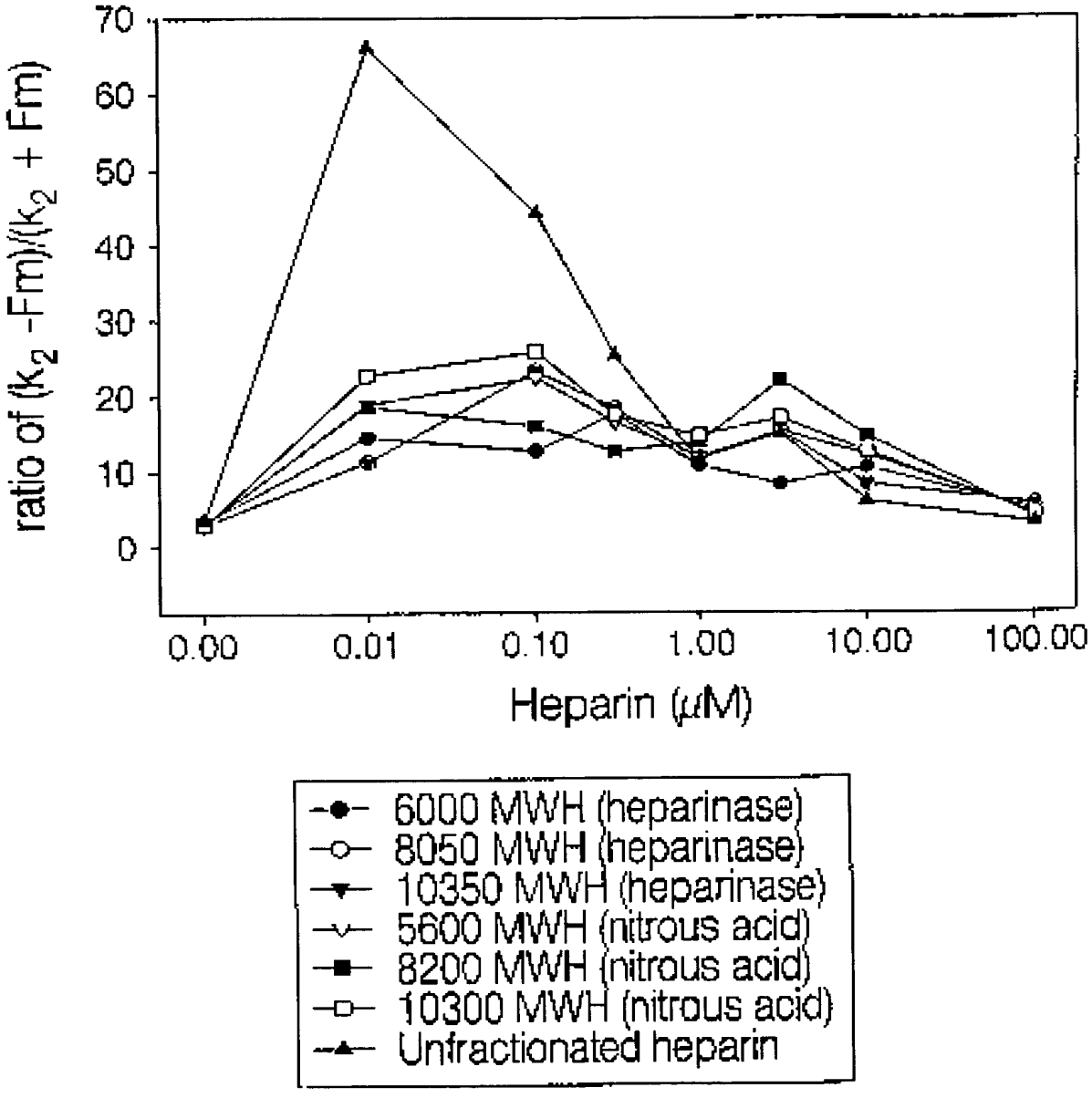


Fig. 32

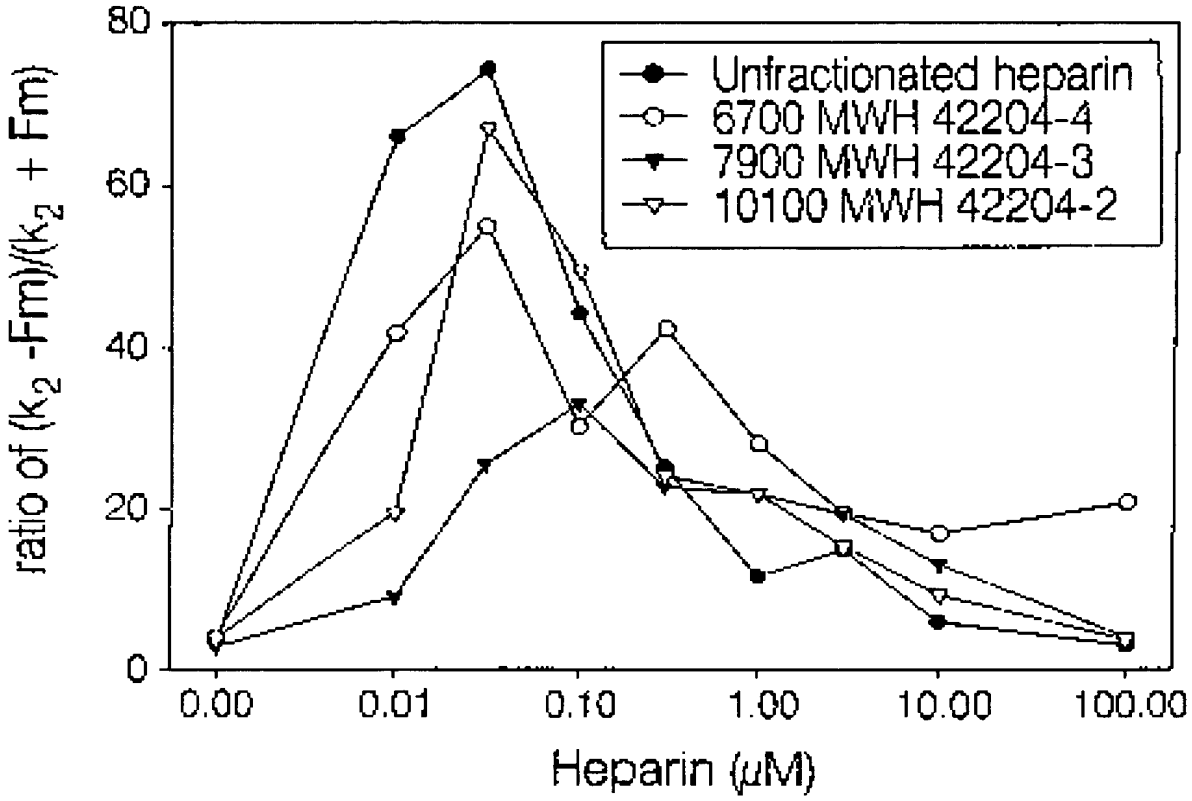


Fig. 33

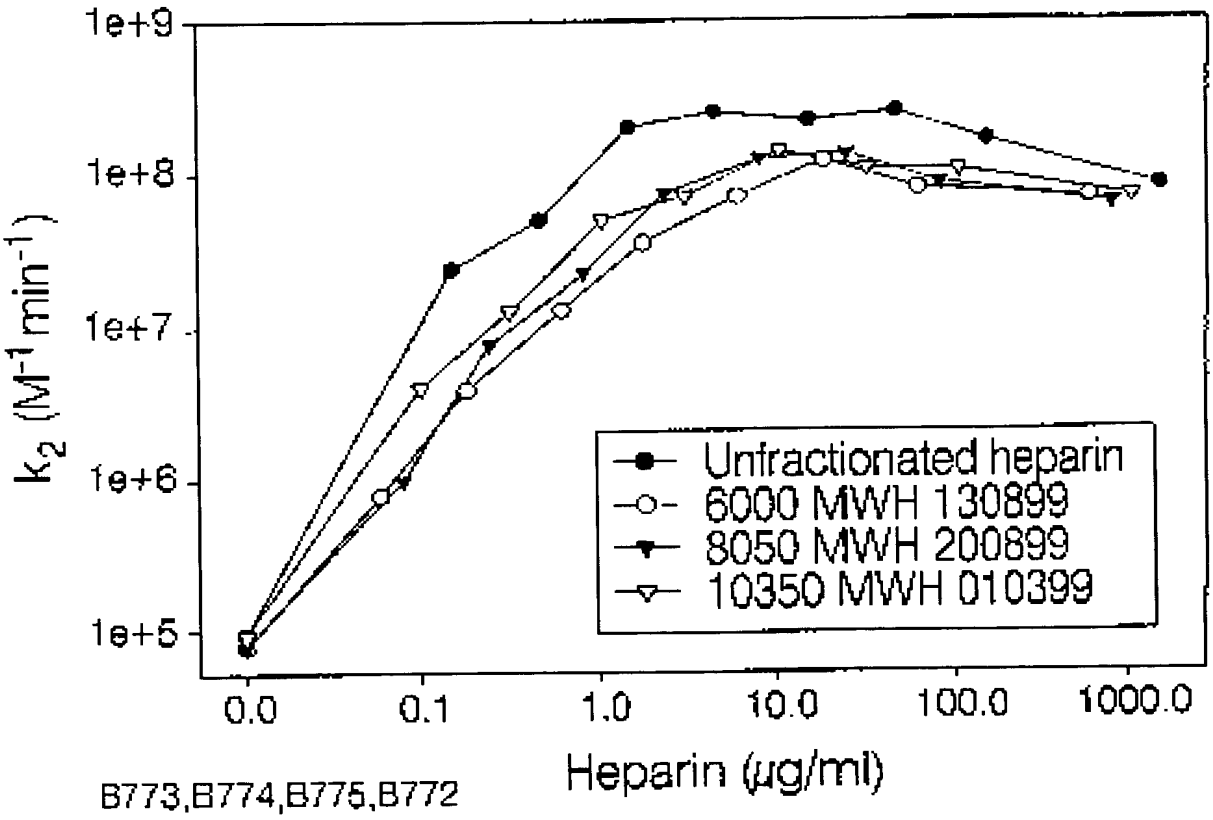


Fig. 34

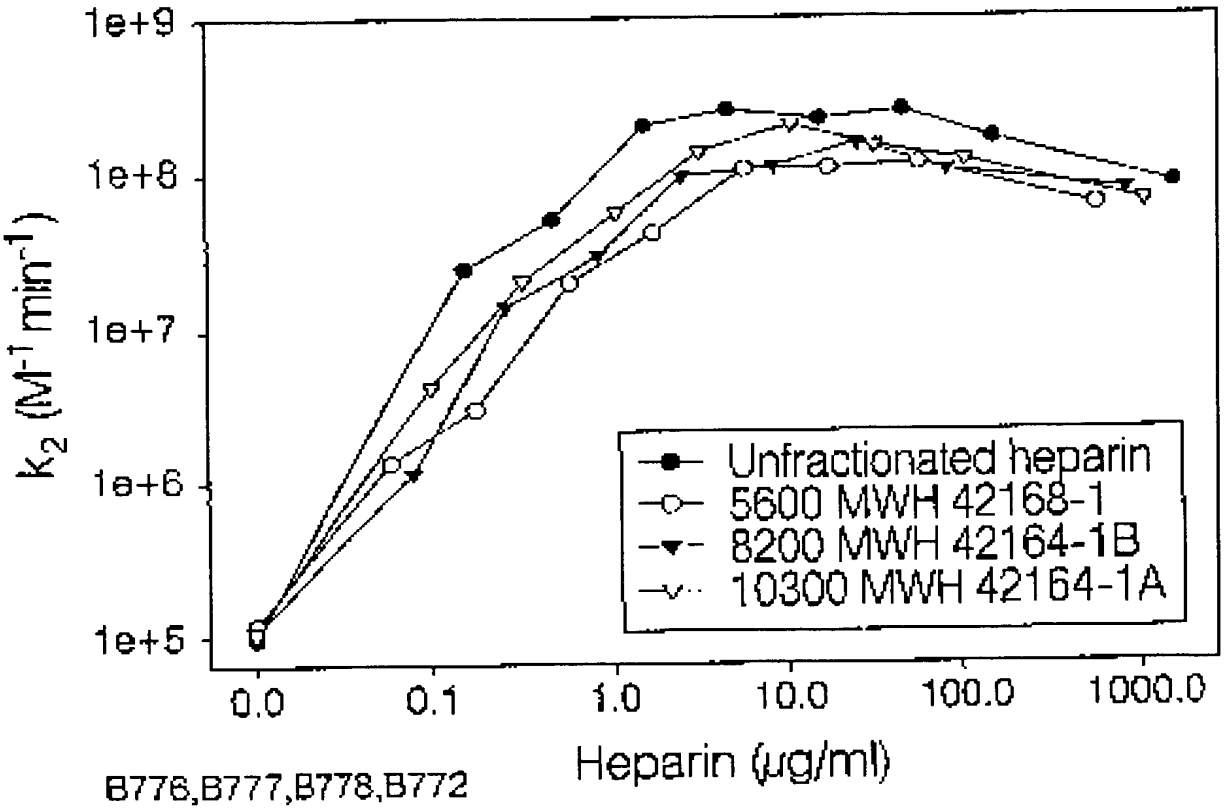


Fig. 35

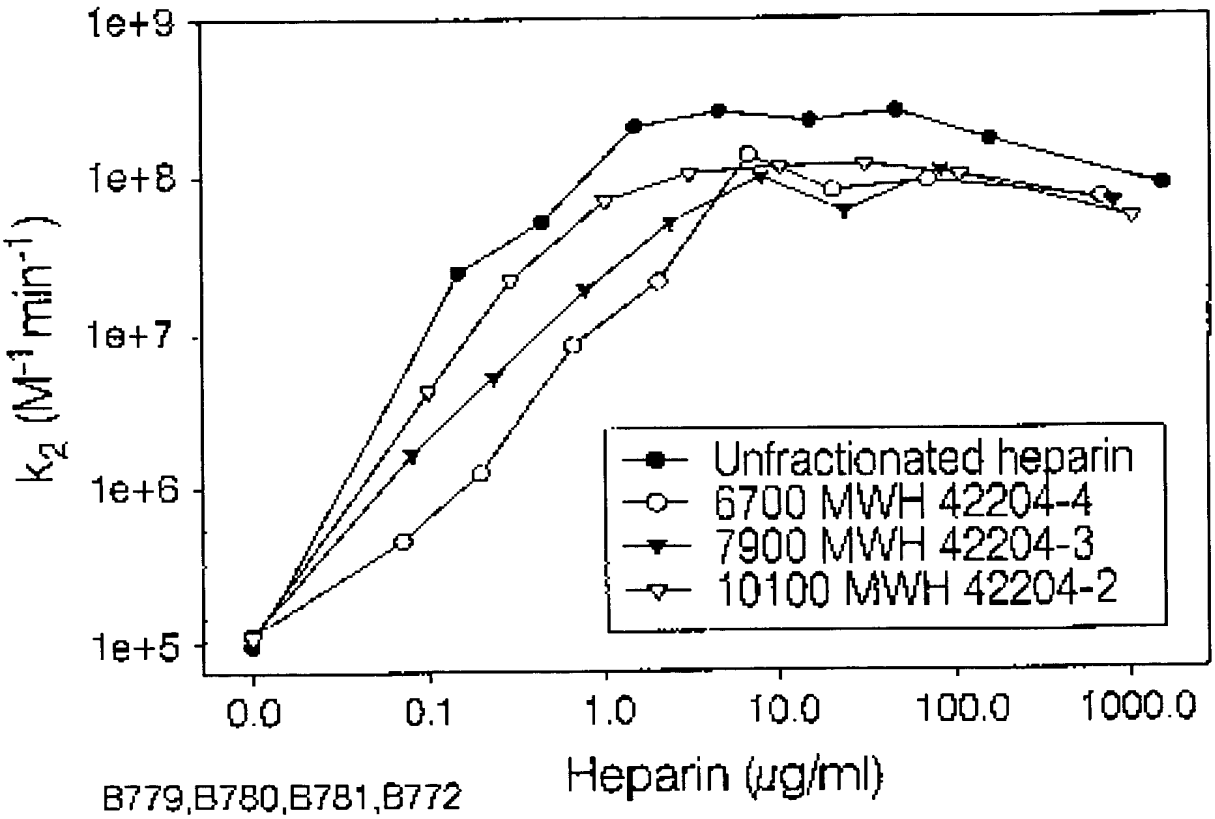


Fig. 36

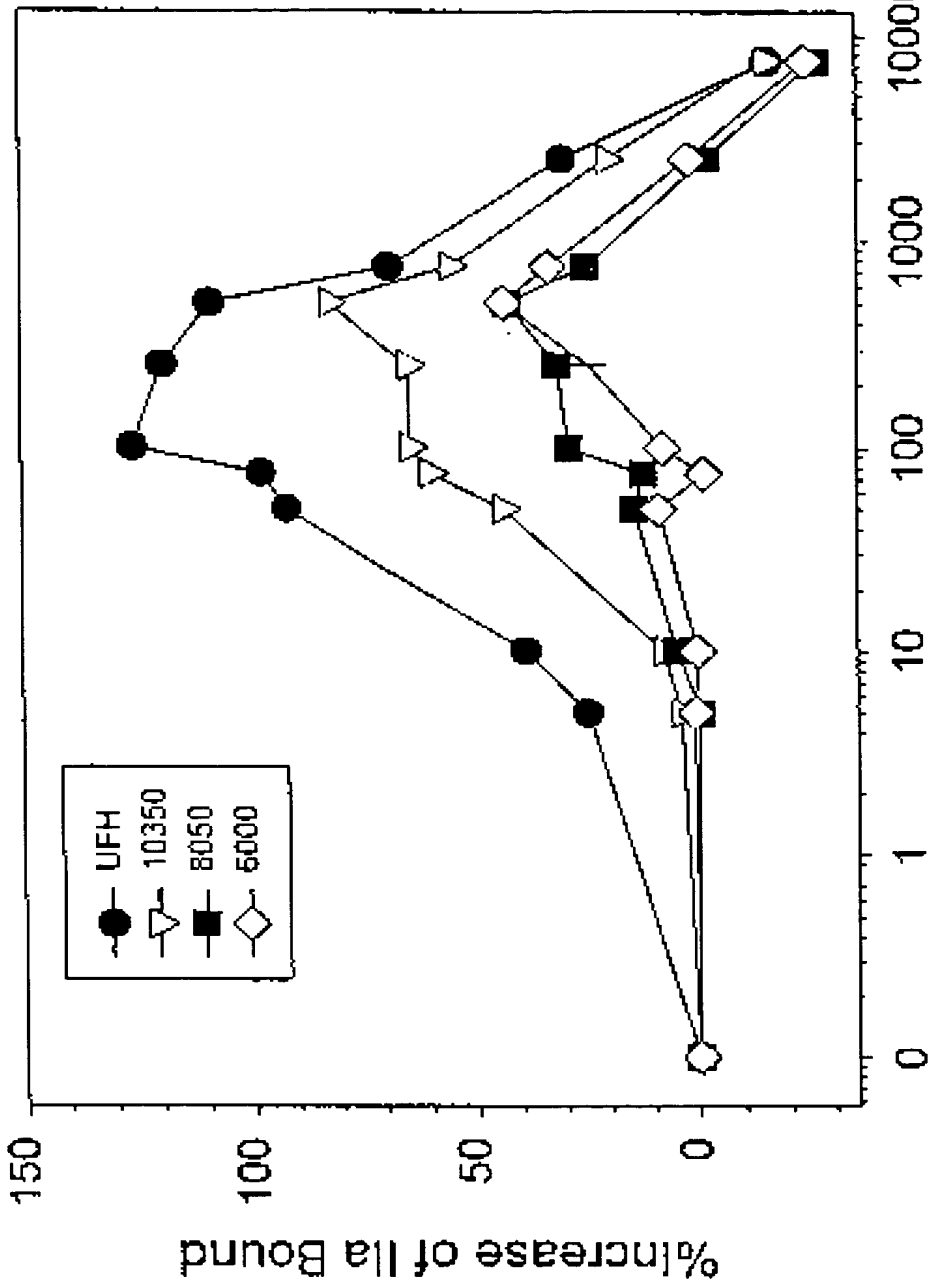


Fig. 37

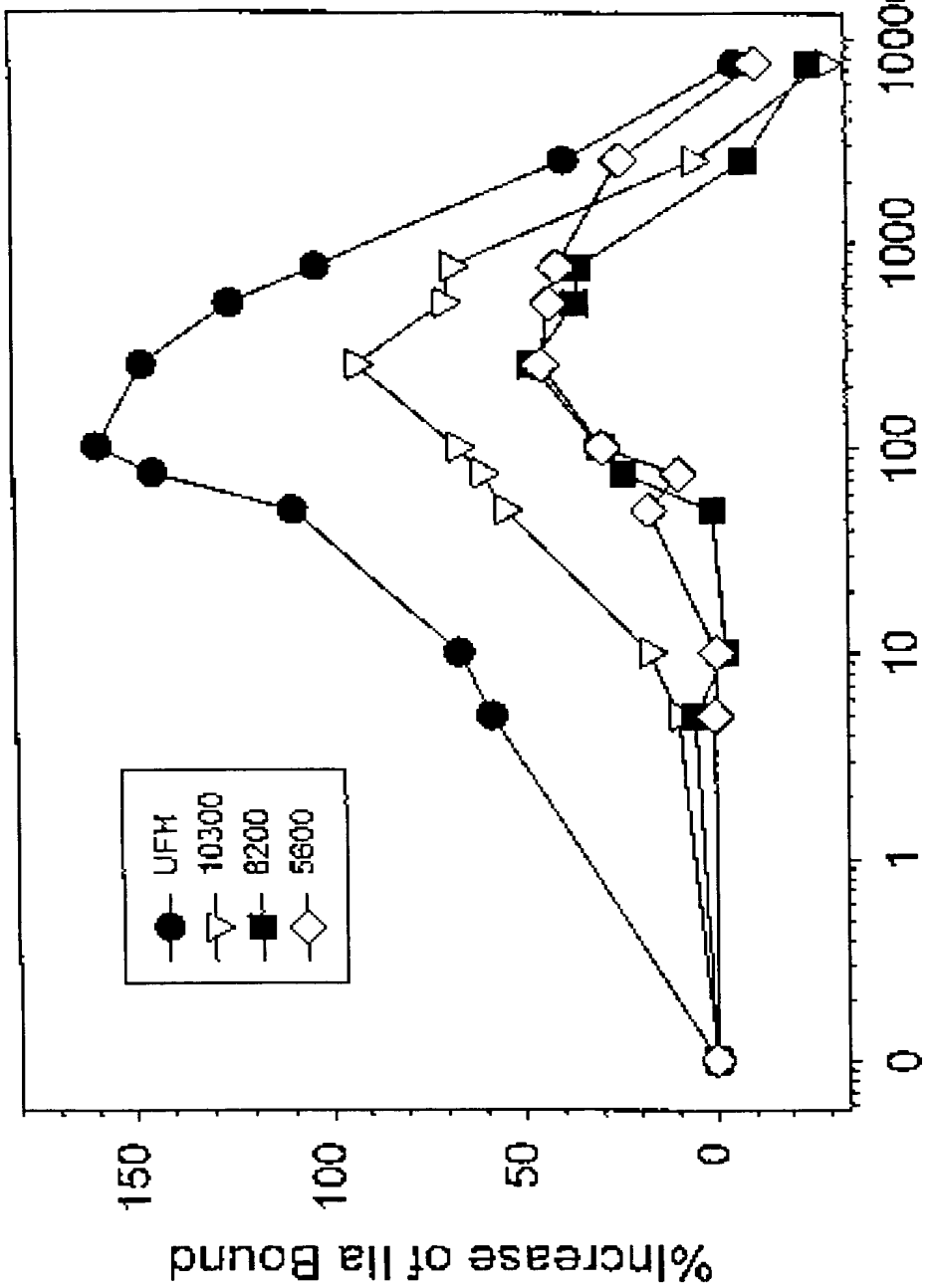


Fig. 38

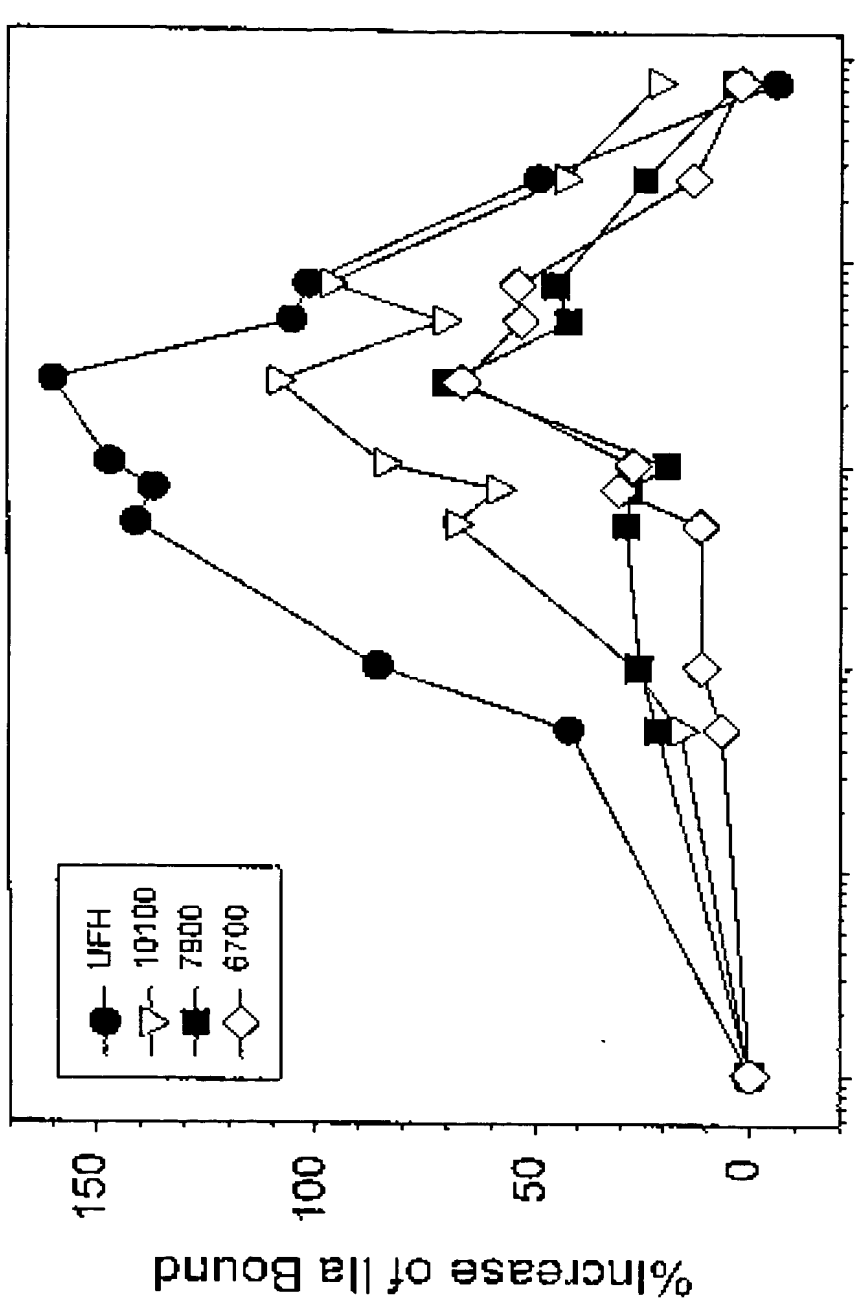


fig. 39

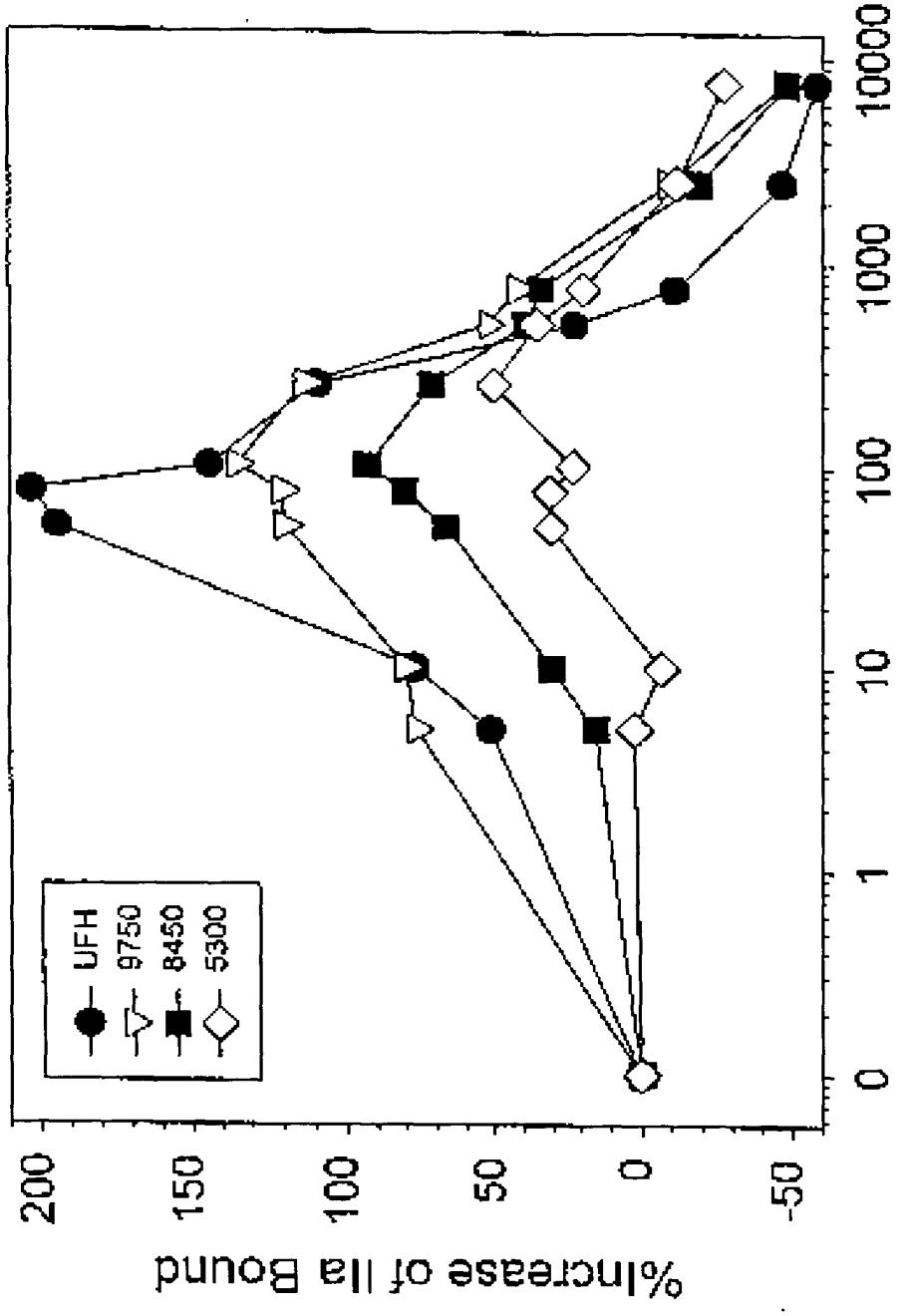


Fig. 40

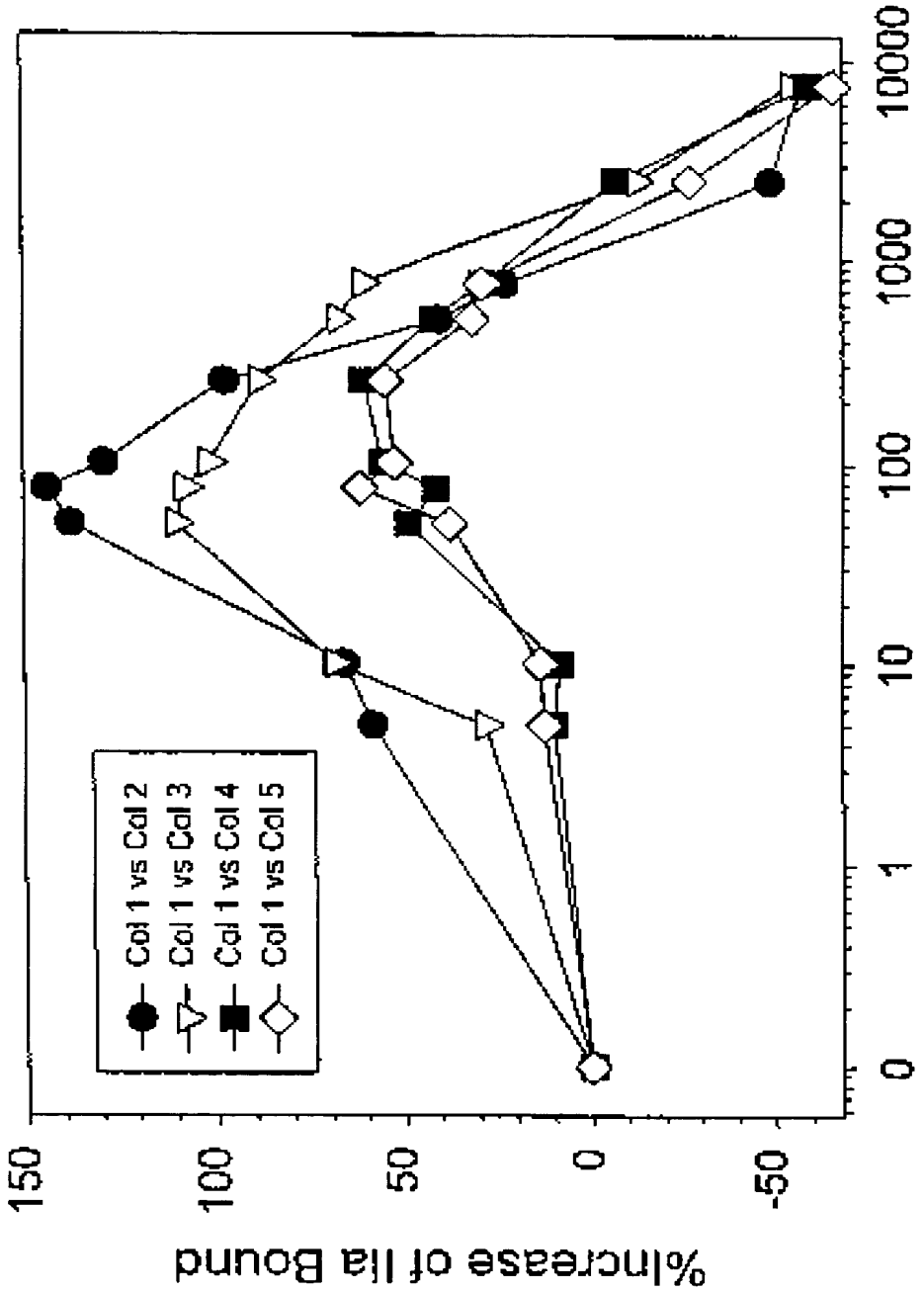


Fig. 41

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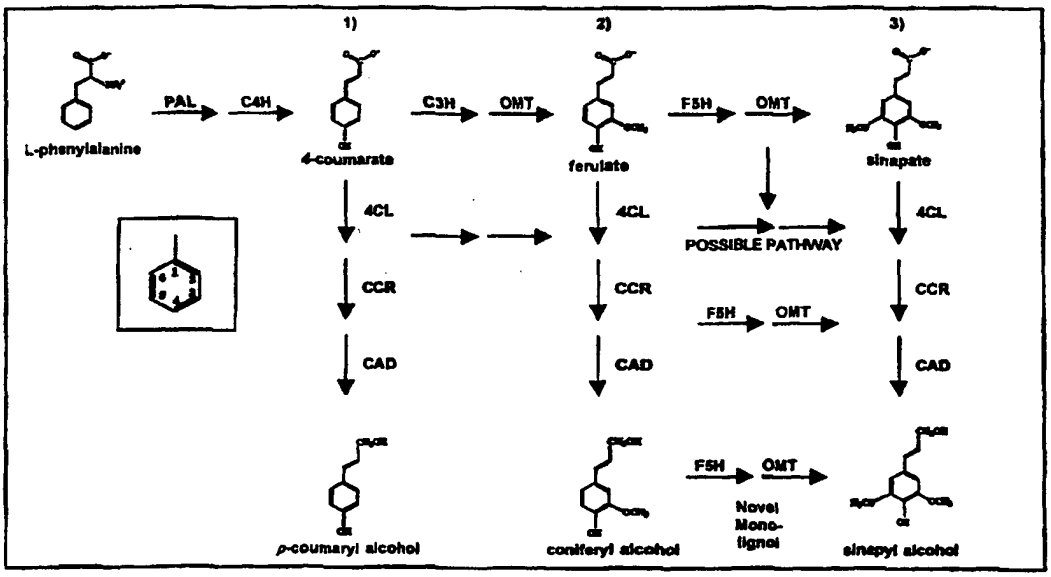

(57) Abstract

A process of producing a transformed gymnosperm plant or plant precursor (cells, callus, embryo, shoot, seed or seedling) having a genome containing at least one expressible transgene that results in modification of the lignin composition of the plant effective to make the plant more commercially desirable. One of the expressible transgenes is preferably the ferulate 5-hydroxylase gene, or a gene which encodes an enzyme that has enzymatic activity substantially similar to the F5H enzyme when transformed in yeast, such that when transformed into plant cells, it results in the production of lignin containing syringyl or other lignin residues in a gymnosperm.

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MODIFICATION OF LIGNIN COMPOSITION OF GYMNOSPERMS

TECHNICAL FIELD

This invention relates to the modification of the lignin composition of gymnosperm species, particularly conifer trees, to make such species more suitable for commercial exploitation.

BACKGROUND ART

Lignin is a cell wall component present in vascular plants that decreases the permeability of cells, contributes to the strength and rigidity of the stem, and protects microfibrils from chemical, physical, and biological attack (Bugos et al. 1991 [4]). [*Note: for full details of references mentioned herein, see the section below headed REFERENCES, the numbers provided in square brackets corresponding to the numbers in that section.*] Despite its advantage to the plant, lignin greatly affects the agro-industrial uses of plants. Lignin content and composition alter the digestibility and dietary conversion of herbaceous crops and are undesirable in the conversion of wood into paper and pulp (Campbell and Sederoff 1996 [6]). Although lignin can contribute up to 25% of the mass of wood, from a pulp and paper viewpoint, lignin does not contribute to the usable biomass in pulping and hence is waste. More importantly, the extraction of lignin during chemical pulping is a costly and difficult process, involving chemical removal. There is a negative correlation between the amount of lignin removed and fiber yield with chemical pulping. Therefore, because the removal of lignin from fibers is a major cost, the modification of both lignin content and composition is a major focus of several research establishments world wide. Of importance is that trees with altered lignin, either decreased content or modified composition to reduce the energy needed to extract the lignin, could allocate more resources to the production of pulpable biomass with decreased costs.

Chemically, lignin is a highly complex network of phenylpropanoid units derived from the oxidative polymerization of one or more of three monolignol precursors which are the end products of the three major branches of the phenylpropanoid pathway (as shown in Figure 1 of the accompanying drawings, introduced the section below headed BRIEF DESCRIPTION OF THE DRAWINGS). As shown in the figure, branch 1 of the pathway yields the monolignol ρ-coumaryl alcohol which makes up the ρ-hydroxyphenyl residue when polymerized into lignin and is present in both angiosperms and gymnosperms. Branch 2 yields the monolignol coniferyl alcohol which makes up the guaiacyl residues when polymerized into lignin and is present in both angiosperms and gymnosperms, yet is the predominant monolignol in gymnosperms. Branch 3 yields sinapyl alcohol which makes up the syringyl residues when polymerized into lignin and is present only in angiosperms, with very few exceptions. These exceptions include reports of syringyl lignin in the gymnosperm species *Podocarpus* and in some species of the *Gnetales*. However, these exceptions are considered rare and are usually not even mentioned in reviews on lignin biosynthesis.

The presence of syringyl residues in angiosperm lignin via branch 3 in the phenylpropanoid pathway accounts for angiosperm lignin being easier to remove during pulping than gymnosperm lignin. One reason syringyl-lignin is easier to remove during pulping, as compared to guaiacyl-lignin produced by gymnosperms, is that the C-5 carbon of the phenyl ring in syringyl-lignin is protected by methoxylation from forming a C5-C5 bond with adjacent monolignol phenyl rings. Once formed, this C-C bond is very difficult to break during delignification and the presence of these bonds accounts for the fact that gymnosperm lignin is harder to pulp than angiosperm lignin.

The inventors of the present invention theorized that if the phenylpropanoid pathway in gymnosperms could be modified such that gymnosperm plants could produce lignin containing syringyl residues, via branch 3, or a modification thereof, of the phenylpropanoid pathway, this would be of great benefit because significant reductions in the pulping costs associated with lignin removal in gymnosperms would be enabled.

However, this requires the creation of an entirely new pathway in gymnosperms, i.e., the creation of the enzymes and substrates in gymnosperm species to enable the branch 3 phenylpropanoid pathway synthesis of syringyl-lignin to proceed through to completion. This is quite different in concept from arranging for over-expression of a gene in an existing metabolic pathway, which is likely to shuttle more metabolites through the pathway, provided other steps do not become limiting.

There are numerous reports on the modification of the phenylpropanoid pathway by genetic engineering. One example is the "sense" suppression of PAL by a bean PAL2 gene in tobacco. These experiments demonstrated that PAL activity becomes rate-limiting to lignin deposition when levels are 3- to 4-fold lower than in wild-type plants (Bate et al. 1994 [2]). While PAL may hold promise for use in engineered lignin modification, it has been suggested that due to its key role in general phenylpropanoid metabolism, the interruption of PAL synthesis would also affect other biochemical pathways. In contrast, the activity of CAD, an enzyme well downstream in the lignin biosynthetic pathway, can be reduced to 10% of normal levels and still have no effect on the quantity of lignin, although clear qualitative differences are observed (Halpin et al. 1994 [12]). From these and other studies on the manipulation by genetic engineering of key enzymes in the lignin biosynthetic pathway (OMT (Dwivedi et al. 1994 [10]; Ni et al. 1994 [16]), F5H (Bell-Lelong et al. 1997, [3]), and peroxidase (Lagrimini et al. 1990

[14])), it is clear that lignin modification is possible. However, such studies also highlight how extremely difficult it is to achieve a change in lignin composition and how it is even more difficult to achieve a change that has commercial relevance. In the CAD antisense work, Halpin et al. [12] reported increased lignin extractability in only 2% of the transformed lines tested. In other words, 98% had no change despite morphological changes such as the appearance of red xylem.

Therefore, these disclosures do not specifically relate to techniques involving genetic engineering to create a lignin which is unique to the plants of interest, i.e. gymnosperms. Firstly, all the published work on the genetic engineering of plants for altered lignin has been done in angiosperms and was done to manipulate an existing endogenous enzyme and biochemical pathway. Even with this, the results were variable, and changing lignin parameters to a level such that they had commercial advantages was difficult. Secondly, the only example of lignin modification in gymnosperms where a gene for a specific enzyme in the phenylpropanoid pathway was down-regulated occurred in a naturally occurring mutant which had virtually no CAD activity (for a review see Whetton et al. 1998 [17]). In this case, genetic engineering was not used and the regulation was again dependent on natural mutation which altered the expression of an endogenous gene.

International patent application PCT/US96/20094, published on July 3, 1997 as WO 97/23599, in the name of Clint Chapple as inventor, and assigned jointly to E.I. Du Pont De Nemours and Company, and Purdue Research Foundation, discloses the nucleotide sequence of a gene encoding an F5H enzyme, the transformation of the genome of plants with the gene, and the resulting modification of lignin composition of the plants. The present application builds on this Chapple application and goes beyond, to describe

the use of this gene, either alone or in conjunction with other genes, to introduce a lignin biosynthetic pathway into gymnosperms.

DISCLOSURE OF THE INVENTION

An object of the invention is to modify gymnosperms by genetic engineering so that modified gymnosperm plants produce lignin of a type that differs from the lignin of wild-type plants of the same species and that is more easily accommodated in commercial utilization of such plants.

Another object of the invention is to modify the lignin precursors in gymnosperms to provide modified monolignol residues, and preferably, a greater content of syringyl residues, or other residues with a side group at the C-5 position of the monolignol ring.

According to one aspect of the present invention, there is provided a process of producing a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild-type plants of the same gymnosperm species, which process comprises: providing a vector containing at least one expressible transgene that results in modification of the lignin composition in the gymnosperm plant; introducing the vector into cells of a gymnosperm plant to produce transformed cells; regenerating transformed gymnosperm callus or shoots from the transformed cells; maturing embryos or plants from the transformed callus or shoots; and generating transformed plant embryos, seeds, seedlings or plants from the matured embryos.

Without wishing to limit the generality of meaning of the term "transgene", we should point out that the term is intended to include foreign DNA (transgenic or introduced genes) that is introduced into a genome of a gymnosperm plant.

According to another aspect of the invention, there is provided a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild-type plants of the same gymnosperm species.

Most preferably, the lignin of the transformed gymnosperm plant contains detectable syringyl residues, or other residues with a side group at the C-5 position of the monolignol ring, whereas the lignin of the wild-type plants contains no detectable syringyl residues or other residues with a side group at the C-5 position of the monolignol ring.

Preferably, the expressible transgenes are genes that code for enzymes required for the lignin biosynthetic pathway, and more preferably the third branch of the pathway by which branch 2 intermediates are converted to sinapyl alcohol. It is therefore to be noted that, in the present invention, at least in its preferred forms, gymnosperm plants are being genetically engineered with genes which encode at least one enzyme that is not normally present in these plants, thereby creating a branch to an existing pathway in gymnosperm plants. The invention therefore differs considerably from prior art procedures that have merely involved the modification of existing pathways in angiosperm plants utililizing enzymes already present in the wild-type plants.

Most preferably, the transgene(s) introduced into the gymnosperm plants includes a ferulate 5-hydroxylase gene, or a transgene that is substantially homologous to said ferulate 5-hydroxylase gene, or a transgene that has an equivalent function, either alone or in conjunction with other genes needed for the biosynthesis of a lignin, i.e. that results in a lignin composition containing syringyl residues. By a "gene that is substantially homologous to said ferulate 5-hydroxylase gene", we mean a gene which can be shown to have ferulate 5-hydroxylase activity in yeast or having at least 50% homology, and more preferably at least 75% homology, to the F5H gene while exhibiting an ability to modify the lignin content of the gymnosperm plant *in vivo*.

The ferulate 5-hydroxylase gene (or equivalent gene) either alone or in conjunction with other genes, are normally operably linked with at least one regulatory sequence, e.g. cauliflower mosaic virus 35S promoter, a promoter for a phenylalanine ammonia lyase gene, a promoter for a p-coumaryl CoA ligase gene, a promoter for cinnamate 4-hydroxylase or other plant promoters capable of controlling expression of plant genes.

The gymnosperm plants produced by the present invention are preferably from the order *coniferales*. Thus, they may be from the *Picea* species (e.g. *Picea glauca, Picea sitchesis*, or *Picea engelmanii*), or from the *Pinus* species (e.g. *Pinus taeda* or *Pinus radiata*).

According to another aspect of the invention, there is provided a biomass derived from a genetically transformed gymnosperm plant, said biomass containing lignin having syringyl residues, or other residues with a side group at the C-5 position of the monolignol ring, and said transformed plant having an untransformed (wild-type) natural plant whose lignin contains no syringyl residues.

A still further aspect of the invention relates to a method of producing cellulose-containing pulp useful for papermaking and the like, which comprises a lignin-containing biomass derived from a gymnosperm plant to produce pulped mass containing lignin, and removing most of said lignin from said pulped mass, characterized in that said gymnosperm plant is a genetically transformed plant producing lignin containing syringyl residues or other residures with a side group at the C-5 position of the monolignol ring.

As will be appreciated from the above, the present invention is capable of producing transformed gymnosperm plants having a modified lignin content that makes gymnosperm plants more attractive on a commercial and industrial scale.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the basic lignin biosynthetic pathway, the enzyme abbreviations being as described in this application, and the suggested induced pathway(s) being highlighted (the inserted box indicates the numbering in the phenyl ring). Note the three branches of the phenylpropanoid pathway (labeled 1), 2) and 3)): branch 1 yields the monolignol ρ-coumaryl alcohol, present in some angiosperms and gymnosperms; branch 2 yields the monolignol coniferyl alcohol, which is present in both angiosperms and gymnosperms yet is the predominant monolignol in gymnosperms; and branch 3 yields sinapyl alcohol predominant and present only in angiosperms, with very few exceptions.

Figure 2 is a graph showing the mean height growth in 1997 from three different transformed lines derived from two different parental genotypes of F5H-transformed and control (non-transformed) interior spruce somatic

seedlings (note F5H 2d-1 and 2d–2 are two replicate sets of somatic seedlings planted 2 weeks apart);

Figure 3 shows the result of a PCR amplification of a 750 bp arabidopsis F5H fragment using the primer pair cc8/cs278 from 14 Putative Transformed Lines (lanes 1-14); a Non-Transformed I1026 Negative Control (lane 15); a Plasmid only p482-F5H = pCC87Positive Control (lane 16); a Plasmid only pBIC-F5H = pBIC20F5H, Control (lane 17); a Blank, no DNA Negative Control (lane 18); and Molecular Weight (MW) Markers (lane 19); and

Figure 4 shows the genomic nucleotide and amino acid sequences of a known Arabidopsis F5H gene and the F5H enzyme that it encodes (as disclosed in Chapple, WO 97/23599).

BEST MODES FOR CARRYING OUT THE INVENTION

As previously noted, the difficulty in lignin removal in all plants is due to the variety of linkages formed between monolignol precursors during lignin polymerization, which linkages account for lignin polymers being highly heterogeneous. This heterogeneity in lignin and the linkages formed during polymerization have a large influence on the pulping characteristics of wood. For example, the presence of the C-5 methoxylated syringyl residues make hardwood lignin easier to hydrolyze during pulping, while a higher proportion of condensed ρ-hydroxyphenyl residues makes softwood hydrolysis more difficult (Campbell and Sederoff 1996 [6]). This is due in part to the unprotected C-5 group characteristic of ρ-hydroxyphenyl and guaiacyl residues typical of softwoods, accounting for the relatively slower delignification rate of the softwoods (Chiang and Funaoka 1990 [8]).

Any modification of this complex polymer requires an understanding of the metabolic pathway. Fortunately, many steps in the lignin biosynthetic pathway are well understood (Davin et al. 1992[9]). The basic phenylpropanoid pathway is shown in accompanying Figure 1. The pathway begins with the conversion of L-phenylalanine to cinnamic acid, by phenylalanine ammonia-lyase (PAL) followed by the conversion of cinnamate to 4-coumarate by cinnamate 4-hydroxylase (C4H). 4-Coumarate has several potential metabolic fates and these account for pathways to the three monolignol precursors. Thus, 4-coumarate can enter into one of the three monolignol branches of the phenylpropanoid pathway shown in Figure 1 as follows:

Branch 1) Present in both angiosperms and gymnosperms where 4-coumarate is activated to 4-coumaryl-CoA in a reaction catalyzed by 4-coumaryl-CoA ligase (4CL), and reduced by hydroxycinnamyl-CoA reductase (CCR) and cinnamyl alcohol: NAD oxidoreductase (CAD) to 4-hydroxycinnamyl alcohol (ρ -coumaryl alcohol), the first of the three monomeric lignin precursors;

Branch 2) Present in both angiosperms and gymnosperms where 4-coumerate is either: A) 3-hydroxylated and 3-O-methylated to form ferulic acid, followed by activation by 4CL, and reduced by CCR and CAD to yield 3-methoxy-4-hydroxycinnamyl alcohol (coniferyl alcohol), the major lignin precursor in conifers; or B) activated to 4-coumarly-CoA which is subsequently 3-hydroxylated and 3-O-methylated to form feruloyl-CoA, which is then reduced by both CCR and CAD to yield 3-methoxy-4-hydroxycinnamyl alcohol; and

Branch 3) Present only in angiosperms where 4-coumarate is modified as in branch 2); however, either: A) ferulic acid undergoes a ring-hydroxylation by ferulate 5-hydroxylase (F5H) and O-methylation by an O-methyltransferase (OMT) to generate sinapic acid, which is reduced to yield sinapyl alcohol, the source of the syringyl residues typical in angiosperms; or B) proceeds through branch 2 to coniferaldehyde and then to 5-hydroxyconiferaldehyde to sinapaldehyde to sinapyl alcohol; or C) proceeds through branch 2 to coniferaldehyde and then to 5-hydroxyconiferyl alcohol to sinapyl alcohol.

The present invention involves the genetic engineering of gymnosperms to introduce one or more functional genes encoding one or more enzymes that results in a modification of the lignin composition of a gymnosperm plant that makes the gymnosperm plant or plant products more commercially desirable. The modification of gymnosperms with genes for any of the enzymes capable of affecting the phenylpropanoid pathway is within the scope of the invention, provided such genes modify the lignin composition of gymnosperm plants to make the plants more commercially desirable. Preferably, the transgene creates a Branch 3 metabolic pathway, or other residues with a side group at the C-5 position of the monolignol ring, and most preferably one of the genes encodes ferulate 5-hydroxylase (F5H). As noted above, this enzyme is thought to be absent in most gymnosperms (with few exceptions) and is one of the key enzymes missing in conifers which accounts for the difference between angiosperm and gymnosperm lignin (Campbell and Sederoff 1996 [6]). The exceptions are very few as previously noted and include reports of syringyl lignin in the non-coniferales gymnosperm species *Podocarpus* and in some species of the *Gnetales*. These exceptions do not, however, detract from the invention as the vast

number of gymnosperms do not produce syringyl lignin and these exceptions are mentioned in a very minor way, if at all in the literature on the subject.

The inventors have been successful in expressing the F5H gene in spruce (a gymnosperm) and have transformed lines containing this transgene in conjunction with other transgenes in the lignin biosynthetic pathway. Since the inventors have demonstrated expression of the F5H gene in spruce, they believe that its expression in other gymnosperm species is predictable since this clearly shows that not only the F5H gene can be expressed in gymnosperms, but also that its expression can modify lignin in plants which do not contain a pathway for syringyl lignin.

Although not conclusive, support that this single enzyme (F5H), either alone or in conjunction with other enzymes, will alter gymnosperm lignin comes from the fact that mutants of the angiosperm *Arabidopsis* which lack this enzyme produce lignin similar in composition to gymnosperms (Chapple et al. 1992), suggesting the lack of this one enzyme, alone or in conjunction with other enzymes, can account for the difference in lignin composition in an angiosperm where a branch of the phenylpropanoid pathway to guaiacyl-containing lignin already exists.

As noted above, the F5H gene is known and described, e.g. in PCT publication WO 97/23599 on July 3, 1997. The disclosure of this publication is specifically incorporated herein by reference. For convenience, the nucleotide sequence of the F5H gene from *Arabidopsis* and the amino acid sequence of the F5H enzyme is shown in Figure 4 of the accompanying drawings.

The F5H gene can be obtained from an angiosperm species, e.g. *Arabidopsis thaliana,* DNA either by polymerase chain reaction (PCR) using

primers designed to the 5' and 3' ends of the published F5H sequence in Figure 4, or by plasmid rescue of the *fah*1 mutant and complementation as was done by Meyer et al. (1996[15]). The PCR amplified product can then be used to identify the native gene from either a genomic or cDNA library and the gene can be subsequently cloned by standard gene cloning techniques. The isolation of the gene by PCR or from the *fah*1 mutant is believed to be within the competence of any person skilled in the art, so that further explanation is unnecessary. Similar techniques can and have been used to isolate other genes in the lignin biosynthetic pathway which can be used in conjunction with an F5H gene to modify lignin in gymnosperms.

Several constructs of the F5H gene were obtained as explained in the PCT publication mentioned above. These constructs include either genomic and cDNA F5H genes controlled by a cauliflower mosaic virus (CaMV) 35S or cinnimate 4-hydroxylase (C4H) promoter, as well as a C4H-GUS construct to test the expression pattern of the C4H promoter, as well as an OMT construct used in conjunction with F5H and a construct containing an F5H-OMT translational fusion. These and other constructs used in this invention are listed below:

pGA482-F5H = pCC87	a pGA482-based vector containing a CaMV 35S-genomic F5H construct;
pBIC-F5H = pBIC20-F5H	a pBIC20-based vector containing an 18kb genomic fragment containing both the F5H promoter and coding region;
pCC98	a pBI121-based vector containing a CaMV 35S-cDNA F5H construct;

pCC223	a pBI101-based vector containing a C4H-GUS construct for xylem directed expression of GUS;
pC4H-F5H = pCC153	a pGA482-based vector containing a C4H-genomic F5H construct; and
pCC99	a pGA482-based vector containing a double-CaMV 35S-genomic F5H construct.
parabOMT	a pUC based vector containing a CaMV 35S-OMT construct used for co-blasting with pCC99.
pF5H-OMT	a pBINPLUS derived vector containing a double-CaMV 35S-F5H-OMT translational fusion.

Starting materials for such vectors are in common use for the construction of plant transformation vectors and are generally available around the world from various labs. The pBI- series is commercially available from Clontech. The pGA482 vector is described in 1987 Methods Enzymol 153:292-305 and is widely used for plant transformation. The pBIC20is a binary cosmid vector described by Meyer et al. 1996, in Genome Mapping in Plants, ed. Paterson, A.H. (Landis Biochemical Press, Austin, TX). Construction of the pGA482-F5H and pBIC20-F5H plasmids are detailed in Meyer et al., 1996, PNAS, 93.6869-6874 and both are available from that source (Chapple). The other F5H and OMT constructs were made using similar techniques.

As seen in the PCT publication mentioned above, the CaMV 35S constructs have been used successfully to modify lignin content in both *Arabidopsis* and tobacco and were included in the present invention to give ectopic expression of the F5H gene in spruce. The C4H promoter constructs should

direct expression to the xylem, the principal target tissue for lignin modification. Because the C4H promoter was isolated from an *Arabidopsis* C4H gene, its expression - as well as the expression of the native C4H gene - in gymnosperms was previously unknown. The OMT constructs were included to ensure, if needed, the O-methylation of the 5-hydroxylated branch 2 intermediates.

To initiate transformation experiments, the plasmids were transformed into *E. coli* and were subsequently purified by CsCl gradient centrifugation. Each plasmid was checked by restriction digest to confirm its identity. Standard procedures were used for coating gold particles with the plasmids and for microprojectile bombardment of spruce somatic embryos. Regeneration of transformed spruce callus was done on a very low level of kanamycin (2–5μg/ml) and embryo maturation was done using routine protocols for spruce.

Over 10,000 spruce embryos were blasted with the various constructs and over 500 transformed seedlings from over 50 transformed lines have been planted in the greenhouse. No abnormal phenotypes or altered growth patterns have been detected in any of the transformed embryogenic callus lines or seedlings. The results of these experiments are summarized in Table 1 below.

Table 1

Information regarding tested constructs

CONSTRUCT	# KANAMYCIN RESISTANT LINES	# CONFIRMED PCR POSITIVE LINES	# CONFIRMED SOUTHERN POSITIVE LINES	# CONFIRMED NORTHERN POSITIVE LINES	# OF LINES WITH SEEDLINGS REGENERATED
pCC87 35S-gF5H	16	16	8	5	18
pBIC20-F5H F5H-gF5H	6	Inconclusive	Inconclusive	Inconclusive	6
pCC98 35S-cF5H	5	2 – Inconclusive	2	nd	1
pCC223 C4H-GUS	6	5	3	ND	5
pCC153 C4H-gF5H	16	10	6	2	12
pCC99 2X 35S-gF5H	20	19	3	17	18
pCC99+ parabOMT	11	Nd	nd	nd	0
Total	80	47	22	24	60

Figure 2 of the accompanying drawings shows the mean height growth of three different F5H transformed lines from two different parental embryogenic genotypes compared with non-transformed somatic seedlings over the growing season. Transformed line I1026 2d is represented by two lots of somatic seedlings planted two weeks apart.

A set of six nested primers for the F5H gene were obtained and tested for amplification of the F5H gene from pCC87.The primer pair consisting of cc8 and cs278 were used to amplify a band of approximately 750bp from the genomic F5H gene.

Figure 3 confirms integration by PCR of the Arabidopsis F5H in 14 different putative F5H transformed embryogenic callus lines (lanes 1–14). A band of approximately 300bp in all lanes including the blank (lane 18) and the non-transformed I1026 control (lane 15), suggests that this fragment is a non-specific amplification product. The band of interest, a 750bp amplification product, is very prominent as expected in the pCC87 plasmid only lane (lane 16) and is absent from both the blank and the non-transformed control. Note the presence of a 750bp band in DNA samples from 10 transformed lines (lanes 1,3,6,7,8,9,11,12,13,14) including the three transformed lines which have somatic seedlings in the greenhouse. The absence of the 750bp band in the remaining putative transformed lines could indicate that these lines are non-transformed escapes, or that the DNA preparation from these lines was poor. This later suggestion is supported by the lack of other background bands in these lanes (lanes 2,4,5,10).

Northern blot analyses have confirmed strong expression of the F5H gene in spruce and Southern blot analysis of transformed lines have conclusively confirmed the PCR results for integration of the inserted DNA into the spruce genome (Table 1).

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The disclosures of all of the above publications are specifically incorporated herein by reference.

CLAIMS:-

1. A process of producing a transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild type plants of the same gymnosperm species, which process comprises:

providing a vector containing at least one expressible transgene that results in modification of the lignin composition in the gymnosperm plant;

introducing said vector into cells of a gymnosperm to produce transformed cells;

regenerating transformed gymnosperm callus or shoots from the transformed cells;

maturing embryos from the transformed callus or shoots; and

generating transformed plant embryos, seeds, seedlings or plants from the matured embryos or shoots.

2. A process according to claim 1, characterized in that said vector is provided with said at least one expressible transgene that encodes at least one enzyme required for the biosynthesis of lignin in the gymnosperm plant.

3. A process according to claim 1, characterized in that said vector is provided with said expressible transgene that encodes at least one enzyme enabling the production of sinapyl alcohol or other residues with a side group at the C-5 position of a monolignol ring during the biosynthesis of lignin.

4. A process according to claim 1, characterized in that said vector is provided with said at least one expressible transgene that encodes at least one enzyme enabling the production of lignin containing syringyl residues or other residues with a side group at the C-5 position of a monolignol ring.

5. A process according to claim 4, characterized in that said vector is provided with an expressible transgene encoding ferulate 5-hydroxylase, or a transgene that has substantially homologous activity to said ferulate 5-hydroxylase gene, either alone or in conjunction with other genes involved in lignin biosynthesis.

6. A process according to claim 5, characterized in that one of the said substantially homologous gene has at least 50% homology with said ferulate 5-hydroxylase gene.

7. A process according to claim 5, characterized in that said substantially homologous gene has at least 75% homology with said ferulate 5-hydroxylase gene.

8. A process according to any preceding claim, characterized in that said gymnosperm plant is from the order coniferales.

9. A process according to any preceding claim, characterized in that said gymnosperm plant is from the species *Picea*.

10. A process according to claim 9, characterized in that said plant is *Picea glauca, Picea sitchesis*, or *Picea engelmanii*.

11. A process according to any one of claims 1 to 8, characterized in that said gymnosperm plant is from the species *Pinus*.

12. A process according to claim 11, characterized in that said gymnosperm plant is *Pinus taeda* or *Pinus radiata*.

13. A process according to claim 5, characterized in that said ferulate 5-hydroxylase gene is operably linked with at least one regulatory sequence.

14. A process according to claim 13, characterized in that said regulatory sequence is cauliflower mosaic virus 35S promoter, a promoter for a phenylalanine ammonia lyase gene, a promoter for a p-coumaryl CoA ligase gene, a promoter for cinnamate 4-hydroxylase, or another plant promoter capable of controlling expression of plant genes.

15. A transformed gymnosperm plant or plant precursor having a genome containing at least one expressible transgene that results in modification of lignin composition in the gymnosperm plant compared to an average lignin composition of untransformed wild type plants of the same gymnosperm species.

16. A gymnosperm plant or plant precursor according to claim 15, characterized in that said plant has a genome containing at least one expressible transgene that encodes at least one enzyme enabling the production of sinapyl alcohol, or other residue with a side group at the C-5 position of a monolignol ring, during the biosynthesis of lignin.

17. A gymnosperm plant or precursor according to claim 15, characterized in that the plant or plant precursor has a genome containing an

expressible transgene that results in a lignin composition containing syringyl residues, or other residue with a side group at the C-5 position of a monolignol ring.

18. A gymnosperm plant or precursor according to claim 15, characterized in that said at least one expressible transgene is a gene encoding ferulate 5-hydroxylase, or a transgene that has substantially homologous activity to said ferulate 5-hydroxylase gene, either alone or in conjunction with other genes involved in lignin biosynthesis.

19. A gymnosperm plant or precursor according to claim 18, characterized in that said substantially homologous gene has at least 50% homology with said ferulate 5-hydroxylase gene.

20. A gymnosperm plant or precursor according to claim 18, characterized in that said substantially homologous gene has at least 75% homology with said ferulate 5-hydroxylase gene.

21. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the order coniferales.

22. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the species *Picea*.

23. A gymnosperm plant or precursor according to claim 22, characterized in that said plant is *Picea glauca, Picea sitchesis*, or *Picea engelmanii*.

24. A gymnosperm plant or precursor according to claim 15, characterized in that said gymnosperm plant is from the species *Pinus*.

25. A gymnosperm plant or precursor according to claim 24, characterized in that said gymnosperm plant is *Pinus taeda* or *Pinus radiata*.

26. A gymnosperm plant or precursor according to claim 18, characterized in that said ferulate 5-hydroxylase gene is operably linked with at least one regulatory sequence.

27. A gymnosperm plant or precursor according to claim 26, characterized in that said regulatory sequence is a cauliflower mosaic virus 35S promoter, a promoter for a phenylalanine ammonia lyase gene, a promoter for a p-coumaryl CoA ligase gene, a promoter for cinnamate 4-hydroxylase, or any other plant promoter capable of controlling expression of plant genes.

28. A biomass derived from a genetically transformed gymnosperm plant, said biomass containing lignin having syringyl residues, or other residue with a side group at the C-5 position of a monolignol ring, and said transformed plant having an untransformed natural wild-type plant whose lignin contains no syringyl residues, or corresponding other residues with a side group at the C-5 position of a monolignol ring.

29. A biomass according to claim 28, resulting from growing and harvesting a genetically transformed plant or plant precursor as defined in any one of claims 15 to 27.

30. A method of producing a cellulose-containing pulp useful for papermaking and the like, which comprises finely dividing a lignin-containing biomass derived from a gymnosperm plant to produce pulped mass containing lignin, and removing at least some of said

lignin from said pulped mass, characterized in that said gymnosperm plant is a genetically transformed plant producing lignin containing syringyl residues or other residues with a side group at the C-5 position of a monolignol ring.

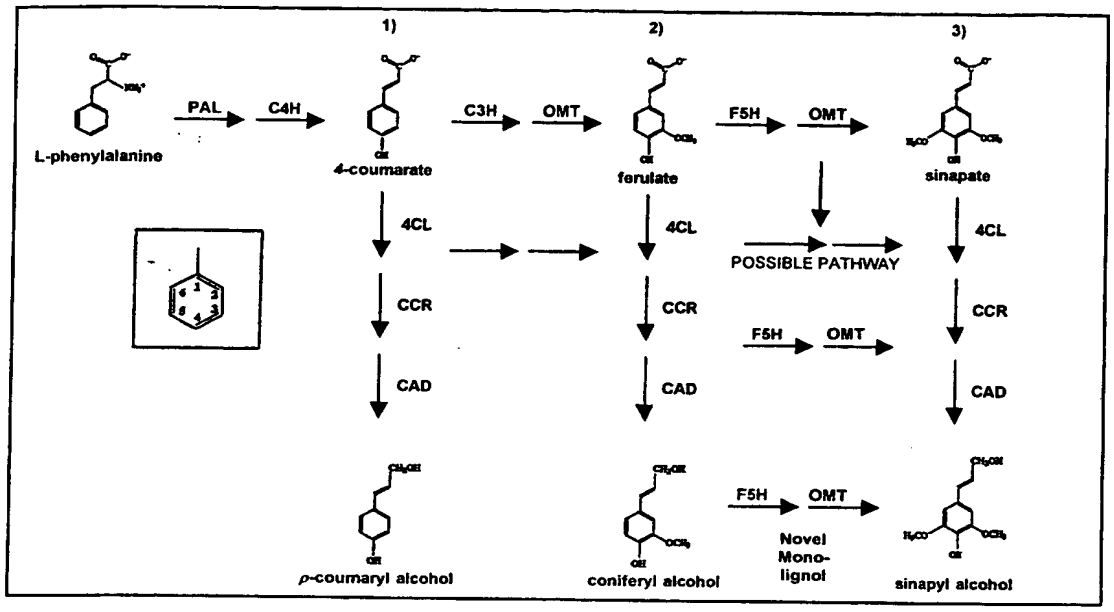
1)
2)
3)
L-phenylalanine
PAL
C4H
4-coumarate
C3H
OMT
ferulate
F5H
OMT
sinapate
4CL
CCR
CAD
POSSIBLE PATHWAY
F5H
OMT
p-coumaryl alcohol
coniferyl alcohol
F5H
OMT
Novel
Mono-
lignol
sinapyl alcohol

Fig. 1

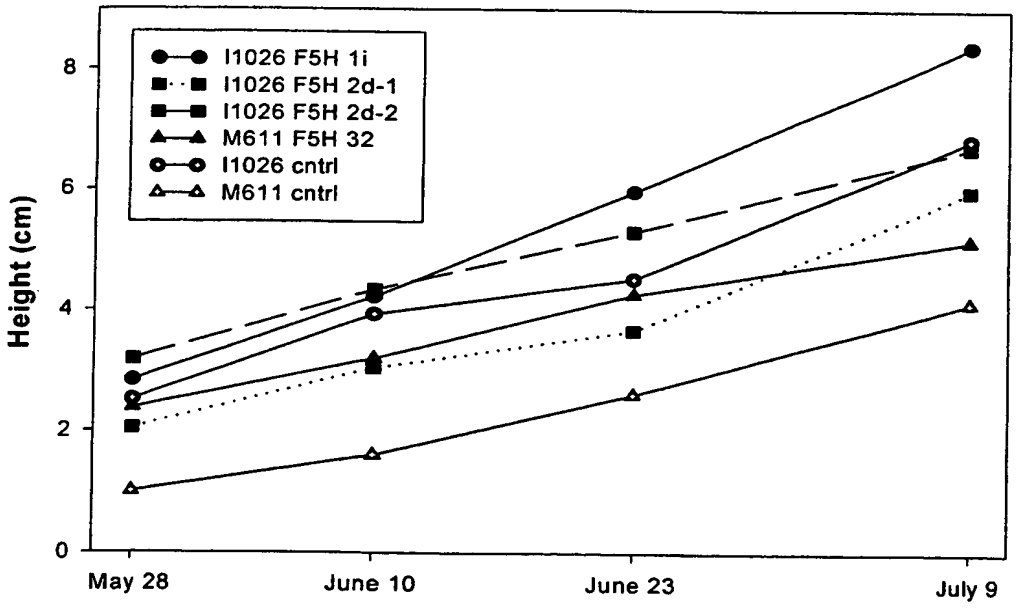
I1026 F5H 1i
I1026 F5H 2d-1
I1026 F5H 2d-2
M611 F5H 32
I1026 cntrl
M611 cntrl
Height (cm)
0
2
4
6
8
May 28
June 10
June 23
July 9

Fig. 2

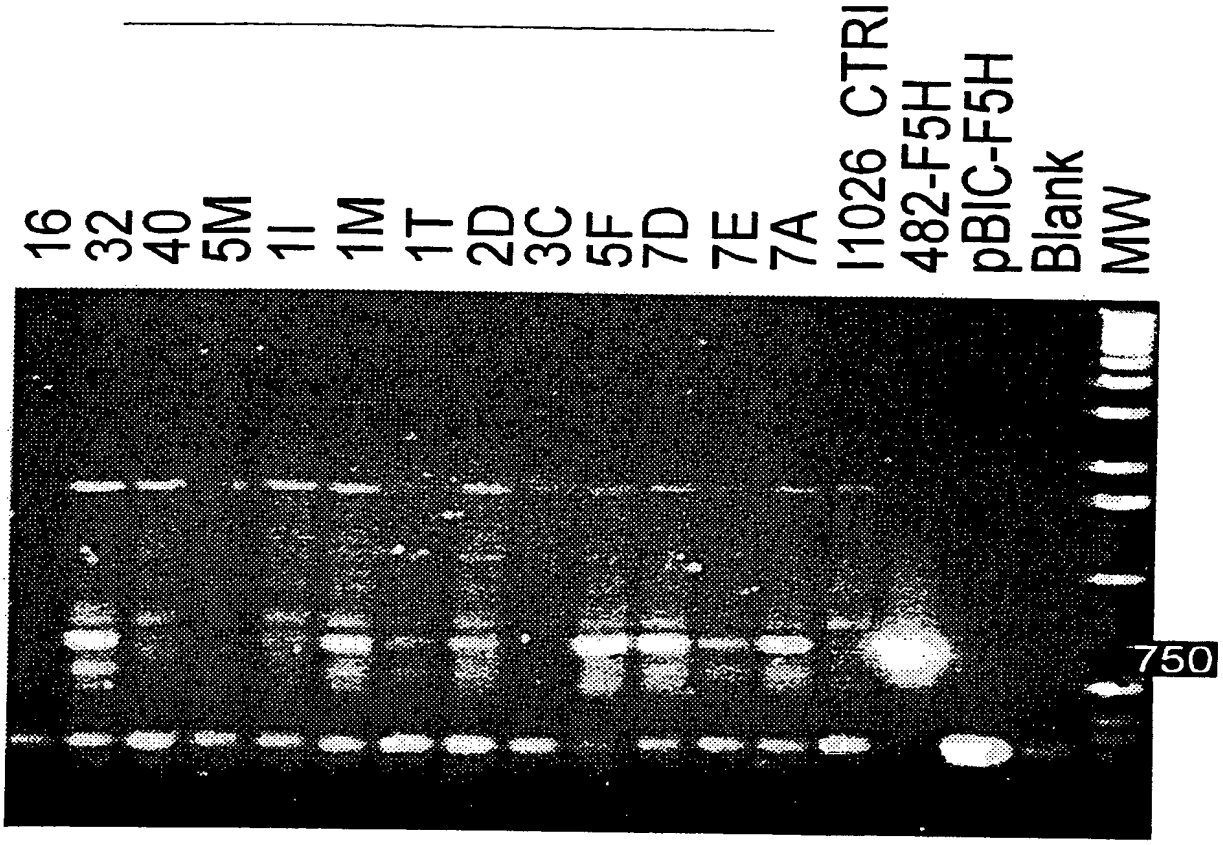
16
32
40
5M
1I
1M
1T
2D
3C
5F
7D
7E
7A
I1026 CTRI
482-F5H
pBIC-F5H
Blank
MW
750

FIG. 3

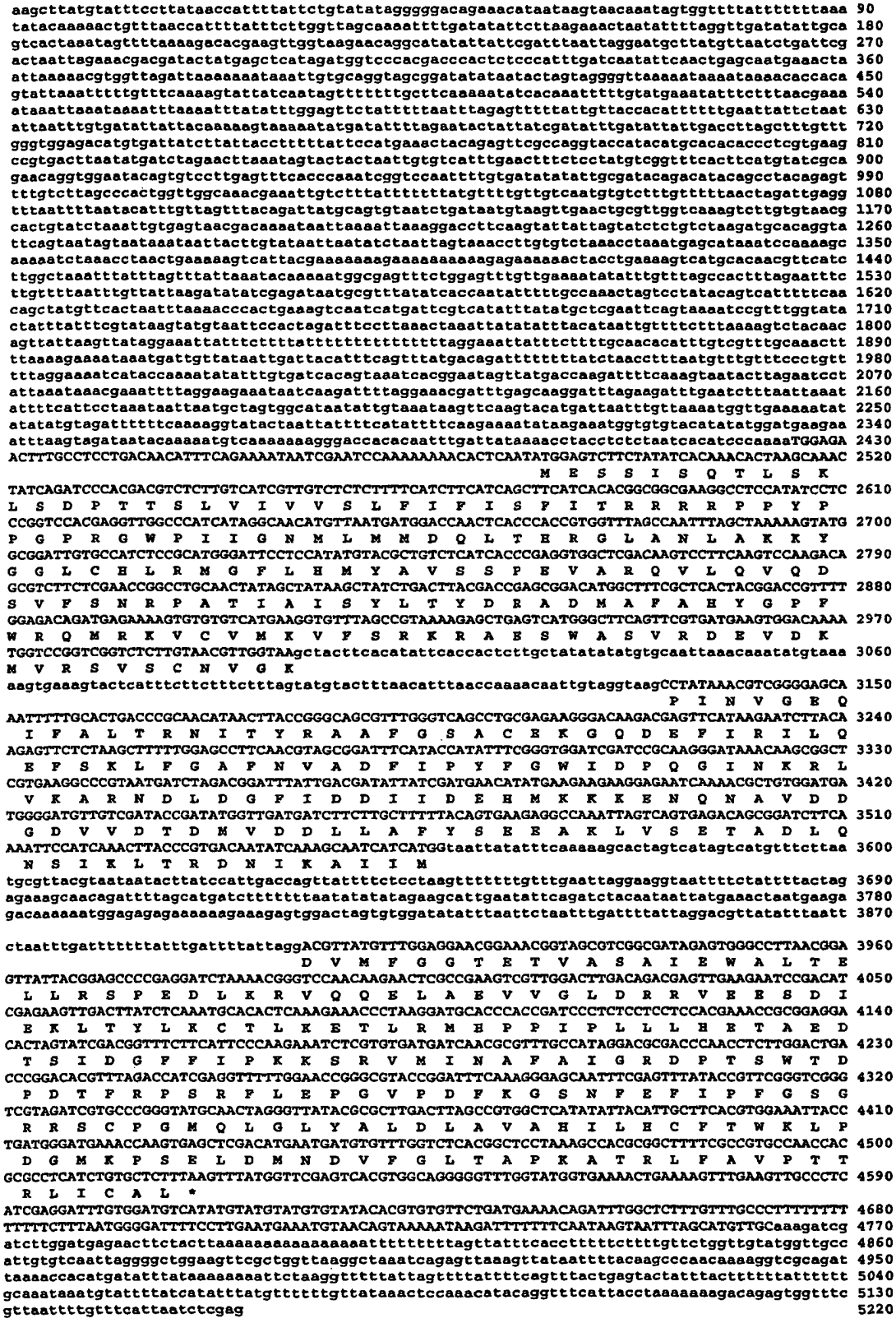

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00074

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 A01H5/00 D21C3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2000	19/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 00/00074

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		CA 2161391 A	27-12-1996
US 5824842 A	20-10-1998	NONE	

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Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

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WO 01/02443 A1

(54) Title: HEPARIN COMPOSITIONS THAT INHIBIT CLOT ASSOCIATED COAGULATION FACTORS

(57) Abstract: The present invention provides compositions and methods for the treatment of cardiovascular diseases. More particularly, the present invention relates to modifying thrombus formation by administering an agent which, *inter alia*, is capable of (1) inactivating fluid-phase thrombin and thrombin which is bound either to fibrin in a clot or to some other surface by catalyzing antithrombin; and (2) inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin III (ATIII). The compositions and methods of the present invention are particularly useful for preventing thrombosis in the circuit of cardiac bypass apparatus and in patients undergoing renal dialysis, and for treating patients suffering from or at risk of suffering from thrombus-related cardiovascular conditions, such as unstable angina, acute myocardial infarction (heart attack), cerebrovascular accidents (stroke), pulmonary embolism, deep vein thrombosis, arterial thrombosis, etc.

TITLE: HEPARIN COMPOSITIONS THAT INHIBIT CLOT ASSOCIATED COAGULATION FACTORS

FIELD OF THE INVENTION

This invention relates generally to compositions and methods for the treatment of cardiovascular disease. More particularly, the present invention relates to modifying thrombus formation and growth by administering a medium molecular weight heparin (MMWH) composition that, *inter alia*, is capable of (1) inactivating fluid-phase thrombin as well as thrombin which is bound either to fibrin in a clot or to some other surface by catalyzing antithrombin; and (2) inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin III (ATIII). In addition, the present invention provides methods and compositions useful for treating cardiovascular disease.

BACKGROUND OF THE INVENTION

Heparin acts as an anticoagulant by binding to antithrombin and markedly increasing the rate at which it inhibits activated factor X (factor Xa) and thrombin. The interaction of heparin with antithrombin is mediated by a unique pentasaccharide sequence that is randomly distributed on about one-third of the heparin chains. To catalyze thrombin inhibition by antithrombin, heparin must bind simultaneously to the enzyme and the inhibitor. Provision of this bridging function requires pentasaccharide-containing heparin chains with a minimum molecular weight of 5,400 Daltons. Even heparin chains of this minimum size may be of insufficient length to bridge thrombin to antithrombin if the pentasaccharide is located in the middle of the heparin chain rather than at either end. In contrast, longer pentasaccharide-containing heparin chains are able to provide this bridging function regardless of the location of the pentasaccharide within the heparin chain.

Like heparin, low molecular weight heparin (LMWH) also acts as an anticoagulant by activating antithrombin. However, with a mean molecular weight of about 4,500 to 5,000 Daltons, the majority of the LMWH chains are too short to bridge thrombin to antithrombin. Consequently, the inhibitory activity of LMWH against thrombin is considerably less than that of heparin.

Although heparin is an efficient inhibitor of fluid-phase thrombin, it is limited in its ability to inactivate thrombin bound to fibrin, *e.g.*, clot-bound thrombin. The resistance of fibrin-bound thrombin to inactivation by the heparin-antithrombin complex reflects the fact that heparin bridges thrombin to fibrin to form a ternary fibrin-thrombin-heparin complex. Formation of this ternary complex heightens the affinity of thrombin for fibrin 20-fold (from a Kd of 3 μM to an apparent Kd of 150 nM). By occupying the heparin-binding site on thrombin, the heparin chain that tethers thrombin to fibrin prevents heparin within the heparin-antithrombin complex from bridging antithrombin to the fibrin-bound thrombin. This explains why fibrin-bound thrombin is protected from inactivation by the heparin-antithrombin complex.

Moreover, with a mean molecular weight of 4,500 to 5,000 Daltons, the majority of the chains of LMWH are also too short to bridge thrombin to fibrin. However, because most of the LMWH chains also are too short to bridge thrombin to antithrombin, LMWH is a poor inhibitor of both fluid-phase and fibrin-bound thrombin.

In view of the foregoing, there still remains a need in the art for improved heparin compositions that are useful, for example, for inhibiting thrombogenesis associated with cardiovascular disease. An ideal heparin composition would be one which can pacify the clot by inactivating fibrin-bound thrombin and by

blocking thrombin generation, thereby preventing the reactivation of coagulation that occurs once treatment is stopped. More particularly, an ideal heparin composition would be one in which the heparin chains are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin to thrombin. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The present invention provides Medium Molecular Weight Heparin (MMWH) compositions comprising heparin chains that are too short to bridge thrombin to fibrin, but that are of a sufficient length to bridge antithrombin to thrombin. Bridging of thrombin to fibrin is only effected by heparin chains that are larger than 12,000 Daltons. Thus, the minimum molecular weight of heparin needed to provide this bridging function is considerably greater than that needed to bridge antithrombin to thrombin. As such, the MMWH compositions of the present invention were designed to fit within this window. With a molecular weight range of about 6,000 to about 12,000 Daltons, the MMWH compositions of the present invention are comprised of heparin chains or sulfated oligosaccharides that are too short to bridge thrombin to fibrin. However, a lower limit of 6,000 Daltons was specifically chosen to ensure that all of the heparin chains of the MMWH compositions are of a sufficient length to bridge antithrombin to thrombin regardless of where the pentasaccharide sequence is located within the heparin chains. For these reasons, the MMWH compositions of the present invention, unlike heparin, inhibit fibrin-bound thrombin and fluid-phase thrombin equally well.

The MMWH compositions of the present invention can pacify the thrombus (or, interchangeably, clot) by inactivating fibrin-bound thrombin, thereby preventing reactivation of coagulation once treatment is stopped, and can block thrombin generation by inhibiting factor Xa. As such, the present invention provides methods of using the MMWH compositions to treat cardiovascular diseases. As explained above, the MMWH compositions of the present invention are a mixture of sulfated oligosaccharides typically having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, from about 8,000 Daltons to about 10,000 Daltons. In a preferred embodiment, the MMMH compounds of the present invention have a mean molecular weight of about 9,000 Daltons. In one embodiment, at least 31% of the MMWH compositions have a molecular weight greater than or equal to 7,800 Daltons. In another embodiment, at least 25% of the MMWH compositions have a molecular weight greater than or equal to 10,000 Daltons. Such MMWH compositions can readily be prepared from standard or unfractionated heparin.

Moreover, the MMWH compositions of the present invention typically have similar anti-factor Xa and anti-factor IIa activities. In a preferred embodiment, the ratio of anti-factor Xa activity to anti-factor IIa activity ranges from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1. In contrast, LMWHs, for example, have significantly more anti-factor Xa activity than anti-factor IIa activity. In a preferred embodiment, the anti-factor Xa activity of the MMWH compositions of the present invention ranges from about 80 U/mg to about 155 U/mg, preferably 90 U/mg to about 150 U/mg and, more preferably, from about 100 U/mg to about 125 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor Xa activity of about 115 U/mg. In a preferred embodiment, the anti-factor IIa activity of the MMWH compositions of the present invention ranges from

about 20 U/mg to about 150 U/mg, preferably 40 U/mg to about 100 U/mg and, more preferably, from about 60 U/mg to about 75 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor IIa activity of about 65 U/mg.

As described above, the MMWH compositions of the present invention comprise heparin chains that are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin to thrombin. Consequently, unlike heparin, the MMWH compositions of the present invention inactivate both fibrin-bound thrombin and free thrombin. Moreover, although most low molecular weight heparin (LMWH) chains are of insufficient length to bridge thrombin to fibrin, they are also too short to bridge antithrombin to thrombin. Consequently, the MMWH compositions of the present invention are considerably better than LMWH at inactivating fibrin-bound thrombin. In addition, although hirudin can inactivate fibrin-bound thrombin, it has no effect on thrombin generation because it is a selective inhibitor of thrombin. Consequently, in contrast to hirudin, the MMWH compositions of the present invention inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin. Thus, by blocking thrombin generation as well as by inhibiting fibrin-bound thrombin, the MMWH compositions of the present invention overcome the limitations of heparin, LMWH and hirudin, particularly in the setting of acute arterial thrombosis.

Selected MMWH compositions of the invention are also contemplated that are enriched for oligosaccharides having an optimal molecular weight range providing particularly advantageous properties as illustrated herein. These MMWH compositions comprise a mixture of oligosaccharides derived from heparin characterized by one, two, three, four, five, or six ,or more of the following characteristics:

(a) having antithrombin- and heparin cofactor II (HCII)-related anticoagulant activity *in vitro;*

(b) the oligosaccharides are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin or HCII to thrombin;

(c) having at least 15%, 20%, 25%, 30%, 35%, or 40% oligosaccharides with at least one or more pentasaccharide sequence;

(d) enriched for oligosaccharides having a molecular weight range from about 6,000 to about 11,000; 7,000 to 10,000; 7,500 to 10,000; 7,800 to 10,000; 7,800 to 9,800; or 7,800 to 9,600; 8,000 to 9,600;

(e) the oligosaccharides have a mean molecular weight of about 7,800 to 10,000, preferably 7,800 to 9,800, more preferably 8,000 to 9,800;

(f) at least 30%, 35%, 40%, 45%, or 50% of the oligosaccharides have a molecular weight greater than or equal to 6000 Daltons, preferably greater than or equal to 8000 Daltons;

(g) a polydispersity of 1.1 to 1.5, preferably 1.2 to 1.4, most preferably 1.3;

(h) having similar anti-factor Xa and anti-factor IIa activities, preferably a ratio of anti-factor Xa activity to anti-factor IIa activity from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1;

(i) an anti-factor Xa activity from about 80 IU/mg to about 155 IU/mg, preferably 90 IU/mg to about 130 IU/mg, more preferably, from about 95 IU/mg to about 120 IU/mg and, most preferably 100-110 IU/mg;

(j) an anti-factor IIa activity from about 20 IU/mg to about 150 IU/mg; preferably 40 IU/mg to about 100 IU/mg, more preferably, from about 80 IU/mg to about 100 IU/mg, most preferably about 90-100 IU/mg.

In accordance with an aspect of the invention a selected MMWH composition of the invention has the characteristics (a), (b), (c) and (d); (a) (b), (c), and (e); (b), (c), (e), and (g); (b), (d), (c), (e), and (h); (b) (c), (d), and (g); (b), (e), (g), (i), and (j); (b), (e), (f), (g), (i) and (j); or (a) through (j).

"Enriched for oligosaccharides" refers to a MMWH composition comprising at least 50%, 55%, 60%, 65%, 70%, 75%, or 80% oligosaccharides within a specified or restricted molecular weight range (e.g. 6,000 to 11, 000; 7,000 to 10,000; 7,800 to 10,000; 7,800 to 9,800; or 8,000 to 9,600).

As a result of their ability to (1) inhibit fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and (2) inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin, the MMWH compositions of the present invention can be used to treat cardiovascular diseases, including unstable angina, acute myocardial infarction (heart attack), cerebral vascular accidents (stroke), pulmonary embolism, deep vein thrombosis, arterial thrombosis, *etc.* As such, the present invention provides methods and pharmaceutical compositions for treating such cardiovascular diseases.

In one embodiment, the present invention provides a method of treating a thrombotic condition in a subject, the method comprising administering to the subject a pharmacologically acceptable dose of a MMWH composition of the invention. The composition may comprising a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, of about 8,000 Daltons to about 10,000 Daltons. In a preferred embodiment, the MMWH composition has a mean molecular weight of about 9,000 Daltons. In another preferred embodiment, the MMWH composition is a selected MMWH composition having an optimal molecular weight range as described herein. In preferred aspects of this embodiment, the thrombotic condition includes, but is not limited to, venous thrombosis (*e.g.*, deep-vein thrombosis), arterial thrombosis and coronary artery thrombosis. In this embodiment, the MMWH composition inhibits thrombus formation and growth, for example, by inhibiting fibrin-bound thrombin and fluid-phase thrombin, and by inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. Preferably, administration of the compounds is achieved by parenteral administration (*e.g.*, by intravenous, subcutaneous and intramuscular injection).

In another embodiment, the present invention provides a method of preventing the formation of a thrombus in a subject at risk of developing thrombosis, the method comprising administering to the subject a pharmacologically acceptable dose of a MMWH composition of the invention. The composition may comprise a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, of about 8,000 Daltons to about 10,000 Daltons. In a preferred embodiment, the MMWH composition has a mean molecular weight of about 9,000 Daltons. In another embodiment, the MMWH composition is a selected MMWH composition having an optimal molecular weight range as described herein. In one aspect of this embodiment, the subject is at increased risk of developing a thrombus due to a medical condition which disrupts hemostasis (*e.g.*, coronary artery disease, atherosclerosis, *etc.*). In another aspect of this embodiment, the subject is at increased risk of

developing a thrombus due to a medical procedure (*e.g.*, cardiac surgery (*e.g.*, cardiopulmonary bypass), catheterization (*e.g.*, cardiac catheterization, percutaneous transluminal coronary angioplasty), atherectomy, placement of a prosthetic device (*e.g.*, cardiovascular valve, vascular graft, stent, *etc.*). In this embodiment, the MMWH compositions can be administered before, during or after the medical procedure. Moreover, administration of the MMWH compositions is preferably achieved by parenteral administration (*e.g.*, by intravenous, subcutaneous and intramuscular injection).

The invention also contemplates the use of a MMWH composition of the invention in the preparation of a medicament for treating a thrombotic condition, or preventing the formation of a thrombus in a subject at risk of developing thrombosis; use of a MMWH composition of the invention in the preparation of a medicament for inhibiting fibrin-bound thrombin and thrombin generation in a subject; use of a MMWH composition of the invention in the preparation of a medicament for treating deep vein thrombosis; and use of a MMWH composition of the invention in the preparation of a medicament for preventing pulmonary embolism in a subject.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate the effects of varying heparin concentrations on thrombin (IIa) binding to fibrin (A) and on thrombin's apparent affinity for fibrin (B).

Figure 2 illustrates the percentage of α-thrombin (α-IIa), γ-thrombin (γ-IIa) or RA-thrombin (RA) that binds to fibrin monomer-sepharose in the absence or presence of heparin.

Figure 3 illustrates the effect of hirugen (Hg), prothrombin fragment 2 (F2) or antibody against exosite 2 (Wab) on thrombin (IIa) binding to fibrin monomer-sepharose in the absence or presence of 250 nM heparin.

Figure 4 illustrates the ternary fibrin-thrombin-heparin complex wherein thrombin (IIa) binds to fibrin (Fn) via exosite 1 and heparin (Hp) binds to both Fn and exosite 2 on IIa.

Figure 5 illustrates the effect of fibrin monomer (Fm) on the rates of thrombin inhibition by antithrombin (■) or heparin cofactor II (●) in the presence of 100 nM heparin. Each point represents the mean of at least 2 separate experiments, while the bars represent the SD.

Figures 6A and 6B illustrate the inhibitory effects of 4 μM fibrin monomer ( ) on the rates of thrombin inhibition by antithrombin (A) or heparin cofactor II (B) in the absence or presence of heparin at the concentrations indicated. Each point represents the mean of at least 2 experiments, while the bars represent the SD.

Figure 7 illustrates the interaction of γ-thrombin (γ-IIa), Quick 1 dysthrombin (Q1-IIa) or RA-IIa with fibrin (Fn) in the presence of heparin (Hp). Non-productive ternary complexes are formed because γ-IIa and Q1-IIa have an altered exosite 1, whereas RA-IIa has reduced affinity for Hp.

Figure 8 illustrates the effect of binary or ternary complex formation on the Km for hydrolysis of N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide by α-thrombin (α-IIa), γ-thrombin (γ-IIa), or RA-thrombin (RA-IIa). Binary complexes include thrombin-fibrin (IIa-Fn), and thrombin-heparin (IIa-Hp), whereas the ternary

complex is thrombin-fibrin-heparin (IIa-Fn-Hp). Each bar represents the mean of at least two experiments, while the lines represent the SD.

Figure 9 illustrates the effect of unfractionated heparin (UFH) and a 6,000 Da heparin fraction (MMWH) on thrombin (IIa) binding to fibrin.

Figure 10 illustrates the inhibitory effects of 4 μM fibrin monomer on the rate of thrombin inhibition by antithrombin (AT) or heparin cofactor II (HCII) in the presence of heparin or a MMWH composition of the present invention. Each bar represents the mean of at least 2 separate experiments, while the lines represent the SD.

Figure 11 illustrates the cumulative patency in % of standard heparin (SH), low molecular weight heparin (LMWH), a MMWH composition of the present invention, and hirudin (HIR) in the prevention model study.

Figure 12 illustrates the effect of standard heparin (SH), low molecular weight heparin (LMWH), a MMWH composition of the present invention, and hirudin (HIR) on cumulative blood loss at 30 minutes.

Figures 13A and 13B illustrate the efficacy of LMWH and a MMWH composition of the present invention, in the arterial thrombosis model (A), and the effect of LMWH and a MMWH composition of the present invention on blood loss (B).

Figure 14 shows comparative effects of a MMWH composition of the present invention and LMWH on APTT.

Figure 15 shows comparative effects of LMWH and a MMWH composition of the present invention on the anti-Xa level.

Figure 16 is a schematic diagram of the procedure.

Figure 17 shows a modified Wessler model Clot Weight by percentage following treatment with a MMWH composition of the present invention.

Figure 18 shows a comparison of LMWH and a MMWH composition of the present invention: Prophylaxis model.

Figure 19 shows a comparison of LMWH and a MMWH composition of the present invention: Prophylaxis model.

Figure 20 shows a modified Wessler model of clot radioactivity by percentage following treatment with a MMWH composition of the present invention.

Figure 21 is a comparison of LMWH and a MMWH composition of the present invention: prophylaxis model.

Figure 22 is a comparison of LMWH and a MMWH composition of the present invention: prophylaxis model.

Figure 23 is a comparison of LMWH and a MMWH composition of the present invention in a treatment model.

Figure 24 is a comparison of LMWH and a MMWH composition of the present invention in a treatment model.

Figure 25 shows a comparison of LMWH and a MMWH composition of the present invention on thrombus accretion.

Figure 26 shows a comparison of LMWH and a MMWH composition of the present invention on thrombus accretion.

Figure 27 shows treatment of DVT in chronic rabbit model clot accretion with a MMWH composition of the present invention.

Figure 28 shows treatment of DVT in chronic rabbit model % change in clot weight with a MMWH composition of the present invention.

Figure 29 is a graph showing rates of AT inhibition of thrombin with heparinase-derived medium molecular weight (MMW) heparins ± 4 μM fibrin monomer.

Figure 30 is a graph showing rates of AT inhibition of thrombin with nitrous acid-derived medium molecular weight (MMW) heparins ± 4 μM fibrin monomer.

Figure 31 is a graph showing rates of AT inhibition of thrombin with periodate-derived medium molecular weight (MMW) heparins ± 4 μM fibrin monomer.

Figure 32 is a graph showing fold inhibition by fibrin monomer of the rate of thrombin inhibition by AT with heparinase and nitrous acid-derived MMW heparins.

Figure 33 is a graph showing fold inhibition by fibrin monomer of the rate of thrombin inhibition by AT with periodate-derived MMW heparins

Figure 34 is a graph showing rates of AT inhibition of Factor Xa with heparinase-derived medium molecular weight heparins.

Figure 35 is a graph showing rates of AT inhibition of Factor Xa with nitrous acid-derived medium molecular weight heparins.

Figure 36 is a graph showing rates of AT inhibition of Factor Xa with periodate-derived medium molecular weight heparins.

Figure 37 is a graph showing the effect of UFH and heparinase-derived medium molecular weight heparins on thrombin binding to fibrin clots.

Figure 38 is a graph showing the effect of UFH and nitrous acid-derived medium molecular weight heparins on thrombin binding to fibrin clots.

Figure 39 is a graph showing the effect of UFH and periodate-derived medium molecular weight heparins on thrombin binding to fibrin clots.

Figure 40 is a graph showing the effect of UFH and size restricted heparinase-derived medium molecular weight heparins on thrombin binding to fibrin clots.

Figure 41 is a graph showing the effect of UFH and size restricted nitrous acid-derived medium molecular weight heparins on thrombin binding to fibrin clots.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The present invention provides Medium Molecular Weight Heparin (MMWH) compounds that (1) inhibit fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and (2) inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin. These MMWH compositions are a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, from about 8,000 Daltons to about 10,000 Daltons. In an embodiment, the MMWH compositions of the present invention have a mean molecular weight of about

9,000 Daltons. In one embodiment, at least 31% of the MMWH compositions have a molecular weight greater than or equal to 7,800 Daltons. In another embodiment, at least 25% of the MMWH compositions have a molecular weight greater than or equal to 10,000 Daltons.

More particularly, the MMWH compositions of the present invention can pacify the intense prothrombotic activity of the thrombus. The prothrombotic activity of the thrombus reflects the activity of fibrin-bound thrombin and platelet-bound activated factor X (factor Xa), both of which are relatively resistant to inactivation by heparin and LMWH. This explains why these agents are of limited efficacy in the setting of arterial thrombosis and why rebound activation of coagulation occurs when treatment is stopped. Moreover, although hirudin can, in contrast to heparin, inactivate fibrin-bound thrombin, it fails to block thrombin generation triggered by platelet-bound factor Xa. The ability of hirudin to inactivate fibrin-bound thrombin explains why direct thrombin inhibitors are superior to heparin for the short-term management of arterial thrombosis. However, any beneficial effects of these agents are rapidly lost once treatment is stopped because they fail to block thrombin generation that is triggered by platelet-bound factor Xa.

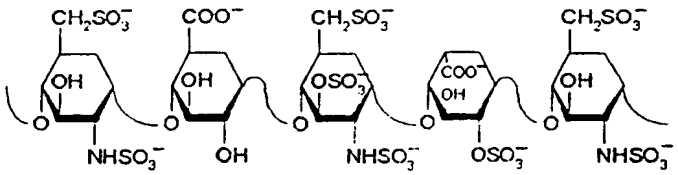
It has now been determined that fibrin-bound thrombin is resistant to inactivation by heparin because the heparin bridges thrombin to fibrin by binding to both fibrin and the heparin-binding site on thrombin with high affinity; the K_d for both the heparin-fibrin and the heparin-thrombin interaction is about 150 nM. Thrombin within this ternary fibrin-thrombin-heparin complex undergoes a conformational change at its active site that likely limits its reactivity with antithrombin. Furthermore, by occupying the heparin-binding site on thrombin, the heparin chain that tethers thrombin to fibrin prevents heparin within the heparin-antithrombin complex from bridging antithrombin to the fibrin-bound thrombin. This explains why thrombin within the ternary fibrin-thrombin-heparin complex is protected from inactivation by heparin or by LMWH chains that are of sufficient length to bridge thrombin to antithrombin. It is likely that a major contributing factor to both the resistance of acute arterial thrombi to these anticoagulants and rebound activation of coagulation after stopping treatment is the inability of heparin, LMWH or hirudin to pacify the intense prothrombotic activity of the thrombus.

In contrast to heparin, LMWH and hirudin, the MMWH compositions of the present invention can pacify the prothrombotic activity of the thrombus by inactivating fibrin-bound thrombin and by inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. More particularly, it has been discovered that the heparin chains of the MMWH compositions of the present invention are too short to bridge thrombin to fibrin, but are of sufficient length to bridge antithrombin to thrombin. Consequently, unlike heparin, the MMWH compositions of the present invention inactivate both fibrin-bound thrombin and free thrombin. Moreover, although most LMWH chains are of insufficient length to bridge thrombin to fibrin, they are also too short to bridge antithrombin to thrombin. Consequently, the MMWH compositions of the present invention are considerably better than LMWH at inactivating fibrin-bound thrombin. In addition, although hirudin can inactivate fibrin-bound thrombin, it has no effect on thrombin generation because it is a selective inhibitor of thrombin. Consequently, in contrast to hirudin, the MMWH compositions of the present invention inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin. Thus, by blocking thrombin generation as well as by inhibiting fibrin-bound thrombin, the

MMWH compositions of the present invention overcome the limitations of heparin, LMWH and hirudin, particularly in the setting of acute arterial thrombosis.

The MMWH compositions of the present invention typically have similar anti-factor IIa and anti-factor Xa activities. In a presently preferred embodiment, the ratio of anti-factor Xa activity to anti-factor IIa activity ranges from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1. In contrast, LMWHs, for example, have significantly more anti-factor Xa activity than anti-factor IIa activity. In a preferred embodiment, the anti-factor Xa activity of the MMWH compositions of the present invention ranges from about 90 U/mg to about 150 U/mg and, more preferably, from about 100 U/mg to about 125 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor Xa activity of about 115 U/mg. In a presently preferred embodiment, the anti-factor IIa activity of the MMWH compositions of the present invention ranges from about 40 U/mg to about 100 U/mg and, more preferably, from about 60 U/mg to about 75 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor IIa activity of about 65 U/mg.

Selected MMWH compositions of the invention are also contemplated that are enriched for oligosaccharides having an optimal molecular weight range providing particularly advantageous properties as illustrated herein. These MMWH compositions comprise a mixture of oligosaccharides derived from heparin characterized by having antithrombin- and heparin cofactor II (HCII)-related anticoagulant activity *in vitro*. The compositions comprise heparin chains that are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin or HCII to thrombin. In particular, the compositions have at least 15%, 20%, 25%, 30%, 35%, or 40% heparin oligosaccharide chains with at least one or more pentasaccharide sequence. "Pentasaccharide sequence" refers to a key structural unit of heparin that consists of three D-glucosamine and two uronic acid residues (See the structure below). The central D-glucosamine residue contains a unique 3-O-sulfate moiety.



The pentasaccharide sequence represents the minimum structure of heparin that has high affinity for antithrombin (Choay, J. et al., Biochem Biophys Res Comm 1983; 116: 492-499). The binding of heparin to antithrombin through the pentasaccharide sequence results in a conformational change in the reactive center loop which converts antithrombin from a slow to a very rapid inhibitor. Consequently, a selected MMWH composition of the invention will be capable of inhibiting fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. Preferably, the selected MMWH compositions of the invention are those that inhibit fibrin-bound thrombin and fluid-phase thrombin equally well.

The selected MMWH compositions comprise oligosaccharides having a molecular weight range from about 6,000 to about 11,000. In accordance with one aspect of the invention a MMWH composition is provided that is enriched for oligosaccharides having a molecular weight range of 7,800 to 8,800, preferably

7,800 to 8,600, more preferably 7,800 to 8,500, most preferably 8,000 to 8,500. In another aspect of the invention a MMWH composition is provided that is enriched for oligosaccharides having a molecular weight range of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most preferably 9,400 to 9,600.

In an embodiment the invention also contemplates a MMWH composition of the invention comprising oligosaccharides with a mean molecular weight of 7,800 to 8,800, preferably 7,800 to 8,600, more preferably 7,800 to 8,500, most preferably 8,000 to 8,500. In another embodiment, the invention contemplates a MMWH composition of the invention comprising oligosaccharides with a mean molecular weight of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most preferably 9,400 to 9,600.

A selected MMWH composition may have a polydispersity of 1.1 to 1.5, preferably 1.2 to 1.4, most preferably 1.3.

A selected MMWH composition of the invention may have similar anti-factor Xa and anti-factor IIa activities. In an embodiment, the ratio of anti-factor Xa activity to anti-factor IIa activity ranges from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1. In a preferred embodiment, the anti-factor Xa activity ranges from about 80 IU/mg to about 155 IU/mg, preferably 90 IU/mg to about 130 IU/mg, more preferably, from about 95 IU/mg to about 120 IU/mg and, most preferably 100-110 IU/mg. In a preferred embodiment, the anti-factor IIa activity ranges from about 20 IU/mg to about 150 IU/mg; more preferably 40 IU/mg to about 100 IU/mg, and most preferably, from about 80 IU/mg to about 100 IU/mg. In an even more preferred embodiment, the compositions have an anti-factor IIa activity of about 90-100 IU/mg.

The MMWH compositions of the present invention can be prepared from low standard or unfractionated heparin or, alternatively, from low molecular weight heparin (LMWH).

In one embodiment, the MMWH compositions of the present invention can be obtained from unfractionated heparin by first depolymerizing the unfractionated heparin to yield lower molecular weight heparin and then isolating or separating out the MMWH fraction of interest. Unfractionated heparin is a mixture of polysaccharide chains composed of repeating disaccharides made up of a uronic acid residue (D-glucuronic acid or L-iduronic acid) and a D-glucosamine acid residue. Many of these disaccharides are sulfated on the uronic acid residues and/or the glucosamine residue. Generally, unfractionated heparin has an average molecular weight ranging from about 6,000 Daltons to 40,000 Daltons, depending on the source of the heparin and the methods used to isolate it. The unfractionated heparin used in the process of the present invention can be either a commercial heparin preparation of pharmaceutical quality or a crude heparin preparation, such as is obtained upon extracting active heparin from mammalian tissues or organs. The commercial product (USP heparin) is available from several sources (*e.g.*, SIGMA Chemical Co., St. Louis, Missouri), generally as an alkali metal or alkaline earth salt (most commonly as sodium heparin). Alternatively, the unfractionated heparin can be extracted from mammalian tissues or organs, particularly from intestinal mucosa or lung from, for example, beef, porcine and sheep, using a variety of methods known to those skilled in the art (see, *e.g.*, Coyne, Erwin, Chemistry and Biology of Heparin, (Lundblad,

R.L., *et al.* (Eds.), pp. 9-17, Elsevier/North-Holland, New York (1981)). In a presently preferred embodiment, the unfractionated heparin is porcine intestinal heparin.

Numerous processes for the depolymerization of heparin are known and have been extensively reported in both the scientific and patent literature, and are applicable to the present invention. Such processes are generally based on either chemical or enzymatic reactions. For instance, a lower molecular weight heparin can be prepared from standard, unfractionated heparin by benzylation followed by alkaline depolymerization; nitrous acid depolymerization; enzymatic depolymerization with heparinase; peroxidative depolymerization, *etc.* Generally methods are chosen that provide compositions with characteristics of a MMWH composition of the invention, in particular a composition of the invention with an optimal molecular weight range. Desired characteristics of a composition of the invention i.e. molecular weight range, mean or average molecular weight, polydispersity, anti-factor Xa activity, anti-factor IIa activity, etc, may be confirmed using standard methods (e.g. see the Examples herein). In a preferred embodiment, a composition of the invention is prepared from unfractionated heparin using nitrous acid depolymerization or heparinase depolymerization.

The unfractionated heparin may be depolymerized by contacting unfractionated heparin, under controlled conditions, to the actions of a chemical agent, more particularly, nitrous acid. The nitrous acid can be added to the heparin directly or, alternatively, it can be formed *in situ*. To generate the nitrous acid *in situ*, controlled amounts of an acid are added to a derivative of nitrous acid. Suitable acids include those which advantageously contain biologically acceptable anions, such as acetic acid and, more preferably, hydrochloric acid. Suitable derivatives of nitrous acid include a salt, an ether-salt or, more preferably, an alkali or alkaline-earth salt. In a presently preferred embodiment, a salt of nitrous acid, a water-soluble salt, more preferably, an alkali salt, such as sodium nitrite ($NaNO_2$), is used.

The depolymerization of unfractionated heparin is preferably carried out in a physiologically acceptable medium, thereby eliminating the problems associated with the use of a solvent that can be detrimental to the contemplated biological applications. Such physiologically acceptable media include, but are not limited to, water and water/alcohol mixtures. In a presently preferred embodiment, water constitutes the preferred reaction medium. In carrying out the depolymerization reaction, it is desirable to use stoichiometric amounts of the reagents (*e.g.*, nitrous acid). The use of stoichiometric amounts of nitrous acid will ensure that when the desired degree of depolymerization is reached, the nitrous acid is entirely consumed. Typically, the weight ratio of unfractionated heparin to sodium nitrite ($NaNO_2$) ranges from about 100 to 2-4 and, more preferably, from about 100 to 3. Using a stoichiometric amount of nitrous acid avoids the need to "quench" a kinetic (ongoing) reaction with, for example, ammonium sulfamate and, in turn, prevents the formation of mixed salts (*e.g.*, sodium and ammonium) of the lower molecular weight heparin intermediates.

In addition, other parameters, such as temperature and pH, are adjusted with respect to one another in order to obtain the desired products under the most satisfactory experimental conditions. For instance, the depolymerization reaction can be carried out at temperatures ranging from about 0° to 30°C. In fact, temperatures lower than 10°C can be used for the production of the desired products. However, in a preferred embodiment, the depolymerization reaction is carried out at ambient temperature, *i.e.*, between

about 20°C and 28°C. Moreover, in a preferred embodiment, the depolymerization reaction is initiated and terminated by first lowering and then raising the pH of the reaction mixture. To initiate the depolymerization reaction, the pH of the reaction mixture is lowered to a pH of about 2.5 to 3.5 and, more preferably, to a pH of about 3.0. Similarly, to terminate the depolymerization reaction, the pH of the reaction mixture is raised to a pH of about 6.0 to 7.0 and, more preferably, to a pH of about 6.75. It should be noted that the progress of the reaction can be monitored by checking for the presence or absence of nitrous ions in the reaction mixture using, for example, starch-iodine paper. The absence of nitrous ions in the reaction mixture indicates that the reaction has gone to completion. The time required for the reaction to reach completion will vary depending on the reactants and reaction conditions employed. Typically, however, the reaction will reach completion in anywhere from about 1 hour to about 3 hours.

Once the reaction has reached completion, the MMWH compositions can be recovered using a number of different techniques known to and used by those of skill in the art. In one embodiment, the MMWH compositions are recovered from the reaction mixture by precipitation, ultrafiltration or chromatography methods. If the desired product is obtained by precipitation, this is generally done using, for example, an alcohol (*e.g.*, absolute ethanol). In a presently preferred embodiment, the MMWH composition is recovered from the reaction mixture using ultrafiltration methods. Ultrafiltration membranes of various molecular weight cuts-offs can advantageously be used to both desalt and define the molecular weight characteristics of the resulting MMWH compositions. Ultrafiltration systems suitable for use in accordance with the present invention are known to and used by those of skill in the art. The commercially available Millipore Pellicon ultrafiltration device is an exemplary ultrafiltration system that can be used in accordance with the present invention. This device can be equipped with various molecular weight cut-off membranes. In a presently preferred embodiment, the resulting MMWH composition is dialyzed or ultrafiltered against purified water (*i.e.*, distilled water (dH_2O)) using a Millipore Pellicon ultrafiltration device equipped with 6,000 Dalton molecular weight cut-off membranes.

After ultrafiltration, the retentate is then lyophilized, *i.e.*, freeze-dried, to give the MMWH composition. The molecular weight characteristics of the resulting MMWH composition can be determined using standard techniques known to and used by those of skill in the art. Such techniques include, for example, GPC-HPLC, viscosity measurements, light scattering, chemical or physical-chemical determination of functional groups created during the depolymerization process, *etc.* In a preferred embodiment, the molecular weight characteristics of the resulting MMWH composition are determined by high performance size exclusion chromatography in conjunction with multiangle laser light scattering (HPSEC-MALLS). Typically, the resulting MMWH composition has an average or mean molecular weight average (Mw) of about 9,000 Daltons. In a selected composition of the invention, the average or mean molecular weight is about 7,800 to 10,000 Daltons, preferably 7,800 to 9,800 Daltons.

Those of skill in the art will readily appreciate that the resulting MMWH compositions can be subjected to further purification procedures. Such procedures include, but are not limited to, gel permeation chromatography, ultrafiltration, hydrophobic interaction chromatography, affinity chromatography, ion exchange chromatography, *etc.* Moreover, the molecular weight characteristics of the MMWH compositions of the pr sent invention can be determined using standard techniques known to and used by those of skill in

the art as described above. As explained, in a preferred embodiment, the molecular weight characteristics of the MMWH compositions of the present invention are determined by high performance size exclusion chromatography in conjunction with multiangle laser light scattering (HPSEC-MALLS).

MMWH compositions of the invention may be prepared by enzymatic depolymerization of heparin by heparinase (see for example, U.S. 3, 766, 167, and U.S. 4,396,762). In accordance with one aspect of the invention, a composition of the invention, particularly a selected composition with an optimal molecular weight range or restricted molecular weight range is prepared by a controlled heparinase depolymerization as described in EP0244236 (Nielsen and Ostergard; No. 87303836.8 published 04.11.87). Using this method a MMWH composition of the invention may be prepared with a desired weight average molecular weight by depolymerizing with heparinase to the corresponding number average molecular weight. The method measures the increase in light absorption (preferably at 230-235 nm i.e. ΔA235) during the course of depolymerization, and depolymerization is stopped when the light absorption has reached a calculated value corresponding to the desired number average molecular weight and the corresponding desired weight average molecular weight.

In another embodiment, the MMWH compositions of the present invention may be obtained by a limited periodate oxidation/hydrolysis of heparin to yield a lower molecular weight heparin, and then isolating or separating out the MMWH fraction of interest. In the first step of this method, heparin is contacted with a limited amount of sodium periodate. In a presently preferred embodiment, the concentration of sodium periodate ranges from about 1 mM to about 50 mM and, more preferably, from about 5 mM to 20 mM. The pH of this reaction mixture ranges from about 3 to 11 and, more preferably, from about 6.5 to about 7.5. The limited periodate oxidation is generally carried out for about 18 hours. In the second step of this method, an alkaline hydrolysis is carried out after the periodate oxidation using metal alkalines, such as NaOH. In a preferred embodiment, the concentration of the metal alkaline, *e.g.*, NaOH, ranges from about 0.1 N to about 1 N and, more preferably, is about 0.25 N. This step is carried out at a temperature ranging from about 0°C to about 50°C and, more preferably, at a temperature of about 25°C, for a time period of about 1 hour to about 10 hours and, more preferably, 3 hours. The desired MMWH compositions are obtained using known methods, such as gel-filtration, ion-exchange chromatography, ultrafiltration, dialysis, quaternary ammonium precipitation, and organic solvent precipitation, as described above. Moreover, the MMWH compositions can be further purified using the methods described above.

Using a limited periodate oxidation/hydrolysis method, a MMWH composition is prepared that is structurally distinct from known LMWH compounds. As described above, in one embodiment, the MMWH compositions of the present invention are prepared by a brief treatment of unfractionated heparin with periodate to yield a product that is oxidized at some of the sulfated uronic acid residues. These oxidized sites may be readily cleaved with base. Consequently, cleavage of the MMWH composition may not be random as is typically the case with the methods currently used to prepare LMWH. Moreover, the 2-0 sulfated uronic acid residues that are susceptible to oxidation by periodate are located with some frequency proximal to pentasaccharide sequences. Consequently, the limited periodate/hydrolysis method of the present invention may result in lower molecular weight heparin chains that have the pentasaccharide sequence

located at the end of the chain which may leave the remainder of the heparin chain long enough to bridge to thrombin.

The MMWH compositions of the present invention are capable of, *inter alia*, (1) inhibiting fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and (2) inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. As such, the MMWH compositions of the present invention can be used to treat a number of important cardiovascular complications, including unstable angina, acute myocardial infarction (heart attack), cerebral vascular accidents (stroke), pulmonary embolism, deep vein thrombosis, arterial thrombosis, *etc.* In a preferred embodiment, the MMWH compositions of the present invention are used to treat arterial thrombosis. As such, in another embodiment, the MMWH compositions of the present invention can be incorporated as components in pharmaceutical compositions that are useful for treating such cardiovascular conditions. The pharmaceutical compositions of the present invention are useful either alone or in conjunction with conventional thrombolytic treatments, such as the administration of tissue plasminogen activator (tPA), streptokinase, and the like, with conventional anti-platelet treatments, such as the administration of ticlopidine, and the like, as well as with intravascular intervention, such as angioplasty, atherectomy, and the like.

The MMWH compositions of this invention can be incorporated into a variety of formulations for therapeutic administration. More particularly, the MMWH compositions of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into various preparations, preferably in liquid forms, such as slurries, solutions and injections. Administration of the MMWH compositions of the present invention is preferably achieved by parenteral administration (*e.g.*, by intravenous, subcutaneous and intramuscular injection). Moreover, the compounds can be administered in a local rather than systemic manner, for example via injection of the compounds directly into a subcutaneous site, often in a depot or sustained release formulation.

Suitable formulations for use in the present invention are found in Remington's Pharmaceutical Sciences (Mack Publishing Company, Philadelphia, PA, 17th Ed. (1985)), the teachings of which are incorporated herein by reference. Moreover, for a brief review of methods for drug delivery, see, Langer, *Science* 249:1527-1533 (1990), the teachings of which are incorporated herein by reference. The pharmaceutical compositions described herein can be manufactured in a manner that is known to those of skill in the art, *i.e.*, by means of conventional mixing, dissolving, levigating, emulsifying, entrapping or lyophilizing processes. The following methods and excipients are merely exemplary and are in no way limiting.

The MMWH compositions of the present invention are preferably formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Generally, pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be

prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

More particularly, for injection, the MMWH compositions can be formulated into preparations by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives, such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives. Preferably, the compositions of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers, such as Hanks's solution, Ringer's solution, or physiological saline buffer.

In addition to the formulations described previously, the MMWH compositions can also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in a therapeutically effective amount. By a "therapeutically effective amount" or, interchangeably, "pharmacologically acceptable dose" or, interchangeably, "anticoagulantly effective amount," it is meant that a sufficient amount of the compound, *i.e.*, the MMWH composition, will be present in order to achieve a desired result, *e.g.*, inhibition of thrombus accretion when treating a thrombus-related cardiovascular condition, such as those described above by, for example, inactivating clot-bound thrombin, inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin, *etc.* The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician. Determination of an effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

A treatment or composition of the invention may be administered to subjects that are animals, including mammals, and particularly humans. Animals also include domestic animals, including horses, cows, sheep, pigs, cats, dogs, and zoo animals.

Typically, the active product, *i.e.*, the MMWH compositions, will be present in the pharmaceutical composition at a concentration ranging from about 2 μg per dose to 200 μg per dose and, more preferably, at a concentration ranging from about 5 μg per dose to 50 μg per dose. Daily dosages can vary widely, depending on the specific activity of the particular MMWH, but will usually be present at a concentration ranging from about 0.5 μg per kg of body weight per day to about 15 μg per kg of body weight per day and,

more preferably, at a concentration ranging from about 1 μg per kg of body weight per day to about 5 μg per kg of body weight per day.

In addition to being useful in pharmaceutical compositions for the treatment of the cardiovascular conditions described above, one of skill in the art will readily appreciate that the active products, *i.e.*, the MMWH compositions, can be used as reagents for elucidating the mechanism of blood coagulation in vitro.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

EXAMPLES

Example 1

Experimental Findings

1.1 Clinical Limitations of Currently Available Anticoagulants:

Heparin, LMWH and direct thrombin inhibitors have limitations in acute coronary syndromes. In patients with unstable angina, there is a clustering of recurrent ischemic events after treatment with these agents is stopped (Theroux, P., *et al.* (1992) *N. Engl. J. Med.* 327:141-145; Granger, C.B., *et al.* (1996) *Circulation* 93:870-888; Oldgren, J., *et al.* (1996) *Circulation* 94 (suppl 1):I-431). This is due to reactivation of coagulation because there is an associated elevation in plasma levels of prothrombin fragments F1.2 (F1.2) and fibrinopeptide A (FPA), reflecting increased thrombin generation and thrombin activity, respectively (Granger, C.B., *et al.* (1995) *Circulation* 91:1929-1935). In patients with acute myocardial infarction, thrombolytic therapy with tissue plasminogen activator (t-PA) or streptokinase induces a procoagulant state characterized by elevated levels of FPA (Eisenberg, P.R., *et al.* (1987) *J. Am. Coll. Cardiol.* 10:527-529; Owen, J., *et al.* (1988) *Blood* 72:616-620), which are only partially reduced by heparin (Galvani, J., *et al.* (1994) *J. Am. Coll. Cardiol.* 24:1445-1452; Merlini, P.A., *et al.* (1995) *J. Am. Coll. Cardiol.* 25:203-209). This explains why adjunctive heparin does not reduce the incidence of recurrent ischemic events in patients receiving streptokinase (Collins, R., *et al.* (1996) *BMJ* 313:652-659), and is of only questionable benefit in those given t-PA (Collins, R., *et al.* (1996) *BMJ* 313:652-659). Although hirudin is better than heparin both as an adjunct to thrombolytic therapy and in patients with non-Q wave infarction who do not receive thrombolytic agents, the early benefits of hirudin are lost within 30 days (GUSTO *Investigators* (1996) *N. Engl. J. Med.* 335(11):775-782). These findings suggest that there is a persistent thrombogenic stimulus that is resistant to both heparin and hirudin.

Similar results are seen in the setting of coronary angioplasty. Recurrent ischemic events occur in 6-8% of patients despite aspirin and high-dose heparin (Popma, J.J., *et al.* (1995) *Chest* 108:486-501). Although hirudin is superior to heparin for the first 72 hours after successful coronary angioplasty, its benefits are lost by 30 days (Serruys, P.W., *et al.* (1995) *N. Engl. J. Med.* 333:757-763). Similarly, at 7 days, hirulog, a semi-synthetic hirudin analogue (Bittl, J.A., *et al.* (1995) *J. Med.* 333:764-769), is better than heparin at preventing recurrent ischemic events in patients undergoing angioplasty for unstable angina after acute myocardial infarction; by 30 days, however, there is no difference between hirulog and heparin (Bittl, J.A., *et al.* (1995) *J. Med.* 333:764-769). It is likely that both the resistance of acute arterial thrombi

to heparin, LMWH and hirudin and the reactivation of coagulation that occurs when treatment is stopped reflect the inability of these anticoagulants to pacify the intense prothrombotic activity of the thrombus.

1.2 Factors Responsible for the Prothrombotic Activity of Acute Arterial Thrombi:

Arterial thrombosis is triggered by vascular injury. Spontaneous or traumatic rupture of atherosclerotic plaque exposes tissue factor which complexes factor VII/VIIa. The factor VIIa/tissue factor complex then initiates coagulation by activating factors IX and X. Although factor VIIa within the factor VIIa/tissue factor complex is rapidly inactivated by tissue factor pathway inhibitor (Broze GJ Jr. (1995) *Thromb. Haemost.* 74:90-93), arterial thrombi remain thrombogenic.

Studies in vitro have attributed the procoagulant activity of arterial thrombi to (a) thrombin bound to fibrin (Hogg, P.J., *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:3619-3623; Weitz, J.I., *et al.* (1990) *J. Clin. Invest.* 86:385-391), or (b) factor Xa (and possibly factor IXa) bound to platelets within the thrombi (Eisenberg, P.R., *et al.* (1993) *J. Clin. Invest.* 91:1877-1883). Fibrin-bound thrombin can locally activate platelets (Kumar, R., *et al.* (1995) *Thromb. Haemost.* 74(3):962-968) and accelerate coagulation (Kumar, R., *et al.* (1994) *Thromb. Haemost.* 72:713-721), thereby inducing an intense procoagulant state. By triggering thrombin generation, platelet--bound factor Xa (and IXa) augments this procoagulant state.

Both fibrin-bound thrombin and platelet-bound factor Xa are resistant to inactivation by heparin and LMWH (Hogg, P.J., *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:3619-3623; Weitz, J.I., *et al.* (1990) *J. Clin. Invest.* 86:385-391; Teitel, J.M., *et al.* (1983) *J. Clin. Invest.* 71:1383-1391; Pieters, J., *et al.* (1988) *J. Biol. Chem.* 263:15313-15318), thereby explaining their inability to pacify the procoagulant activity of acute arterial thrombi. Hirudin can inactivate fibrin-bound thrombin (Weitz, J.I., *et al.* (1990) *J. Clin. Invest.* 86:385-391), but fails to block thrombin generation triggered by platelet-bound clotting factors. In support of this concept, hirudin reduces the levels of FPA, but has no effect on F1.2 levels in patients with unstable angina (Granger, C.B., *et al.* (1995) *Circulation* 91:1929-1935).

There is mounting evidence that both fibrin-bound thrombin and platelet-bound factor Xa contribute to the intense procoagulant activity of thrombi. Thus, the ability of a washed plasma clot to accelerate coagulation when incubated in unanticoagulated whole blood cannot be blocked by either hirudin or tick anticoagulant peptide (TAP), a direct inhibitor of factor Xa that unlike heparin and LMWH inactivates platelet-bound factor Xa as well as free factor Xa (Waxman, L., *et al.* (1990) *Science* 248:593-596). In contrast, a combination of hirudin and TAP abolishes the procoagulant activity of plasma clots, suggesting that pacification of acute arterial thrombi requires agents that not only inhibit fibrin-bound thrombin, but also block thrombin generation triggered by platelet-bound factor Xa. Development of these agents requires an understanding of the mechanisms by which fibrin-bound IIa and platelet-bound factor Xa are protected from inactivation by heparin, LMWH and hirudin.

1.3 Mechanisms by Which Fibrin-bound Thrombin is Protected from Inactivation by Heparin:

Studies indicate that thrombin binding to fibrin is more complex in the presence of heparin than in its absence, and the consequence of thrombin/fibrin interactions has now been better delineated.

1.3.1 Thrombin/Fibrin Interactions in the Absence of Heparin:

In the absence of heparin, α--thrombin binds to fibrin with a Kd = 2 μM. Binding is mediated by exosite 1, the substrate-binding site on thrombin (Fenton, J.W. II, *et al.* (1988) *Biochemistry* 27:7106-7112)

because γ-thrombin (a degraded form of thrombin in which exosite 1 is cleaved) and Quick 1 dysthrombin (a naturally occurring thrombin mutant with Arg 67 within exosite 1 replaced by Cys) fail to bind, whereas RA-thrombin (an exosite 2 mutant (Ye, J., *et al.* (1994) *J. Biol. Chem.* 269:17965-17970) with decreased affinity for heparin because Arg residues 93, 97, and 101 are replaced by Ala) binds to fibrin with an affinity similar to that of α-thrombin.

1.3.2 Thrombin/Fibrin Interactions in the Presence of Heparin:

When heparin is present, the amount of thrombin that binds to fibrin changes, as does the mode of thrombin interaction with fibrin. With heparin concentrations up to 250 nM, the amount of thrombin that binds to fibrin increases (Figure 1A) as does the apparent affinity of thrombin for fibrin (Figure 1B); at higher heparin concentrations, however, thrombin binding (Figure 1A) and the affinity of thrombin for fibrin progressively decrease (Figure 1B). These data extend the results of Hogg and Jackson who demonstrated enhanced thrombin binding to fibrin with fixed concentrations of heparin (see, Hogg, P.J., *et al.*, *J. Biol. Chem.* 265:241-247 (1990)).

The mode of thrombin binding also changes in the presence of heparin. Whereas thrombin binds to fibrin via exosite 1 in the absence of heparin, enhanced α-thrombin binding seen in the presence of heparin is mediated by exosite 2 because heparin augments the binding of γ-thrombin to the same extent as α-thrombin but has little effect on the binding of RA-thrombin (Figure 2). Furthermore, excess α-thrombin bound in the presence of heparin is displaced with an antibody to exosite 2 or with prothrombin fragment 2 (F2) which, like heparin, also binds to exosite 2 (Arni, R.K., *et al.* (1993) *Biochemistry* 32:4727-4737). In contrast, hirugen, a synthetic analogue of the C-terminal of hirudin (Maraganore, J., *et al.* (1989) *J. Biol. Chem.* 264:8692-8698), has no effect on heparin-dependent binding of thrombin (Figure 3).

Such findings are interpreted as indicating ternary fibrin-thrombin-heparin complex formation wherein thrombin binds to fibrin directly via exosite 1, and heparin binds to both fibrin and exosite 2 on thrombin (Figure 4). This occurs because the affinity of heparin for fibrin (Kd = 180 nM) is similar to its affinity for α-thrombin (Kd = 120 nM). Heparin's interaction with fibrin is pentasaccharide--independent because heparin chains with low affinity for antithrombin bind as tightly as high affinity chains. The biphasic effect of heparin on thrombin binding (Figure 1) supports the concept of ternary complex formation. Thus, heparin promotes thrombin binding to fibrin until the heparin binding sites are saturated. With higher heparin concentrations, thrombin binding decreases as nonproductive binary fibrin-heparin and thrombin-heparin complexes are formed.

1.3.3 Consequences of Thrombin/Fibrin Interactions:

Thrombin within the ternary fibrin--thrombin-heparin complex is protected from inactivation by both antithrombin and heparin cofactor II (HCII). HCII is a naturally occurring antithrombin found in plasma that serves as a secondary inhibitor of thrombin. Thus, the heparin-catalyzed rate of thrombin inactivation by antithrombin or HCII is decreased in the presence of fibrin monomer (Figure 5). Over a wide range of heparin concentrations, the rates of inactivation by antithrombin and HCII in the presence of saturating amounts of fibrin monomer are up to 60- and 250-fold slower, respectively, than they are in its absence (Figures 6A and 6B). For protection to occur, both exosites must be occupied; exosite 1 by fibrin and exosite 2 by heparin. Thus, even though heparin enhances the binding of γ-thrombin and Quick 1

dysthrombin to fibrin by binding to their intact exosite 2 and bridging them to fibrin, neither is protected from inactivation because their altered exosite 1 fails to interact with fibrin (Figure 7). RA-thrombin is susceptible to inactivation because even though it binds to fibrin with an affinity similar to that of α--thrombin, it has reduced affinity for heparin because of mutations at exosite 2 (Figure 7).

1.3.4 Evidence that Thrombin Within the Ternary Fibrin-Thrombin-Heparin Complex Undergoes Allosteric Changes at the Active Site:

Allosteric changes in the active site of thrombin induced by ternary complex formation likely reduce thrombin reactivity with its substrates or inhibitors. In support of this concept, it has been shown that the rate of thrombin-mediated cleavage of a synthetic substrate is increased when IIa is bound within the ternary fibrin-thrombin-heparin complex, but not with binary thrombin-heparin or thrombin-fibrin complexes (Figure 8).

Example 2

2.0 Development of Medium Molecular Weight Heparin:

To catalyze thrombin inhibition, heparin bridges antithrombin to thrombin (Danielsson, A., *et al.* (1986) *J. Biol. Chem.* 261:15467-15473). Provision of this bridging function requires heparin chains with a minimal molecular weight of 5,400 (Jordan, R.E., *et al.* (1980) *J. Biol. Chem.* 225:10081-10090). Because the majority of LMWH molecules are < 5,400 Da, LMWH has little inhibitory activity against thrombin (Jordan, R.E., *et al.* (1980) *J. Biol. Chem.* 225:10081-10090). Since heparin bridges thrombin to fibrin to form the ternary fibrin-thrombin-heparin complex, it was hypothesized that this function also requires heparin chains of minimum molecular mass. Further, it was postulated that if this minimum molecular mass is different from that needed to bridge antithrombin to thrombin, there may be a window wherein the heparin chains are too short to bridge thrombin to fibrin, but are of sufficient length to bridge antithrombin to thrombin, thereby overcoming an important mechanism of heparin resistance.

It has now been discovered that such a window exists. For instance, the MMWH compositions of the present invention are long enough to catalyze thrombin inhibition by antithrombin, but do not promote thrombin binding to fibrin (Figure 9). In contrast to heparin, therefore, the rate of MMWH-catalyzed thrombin inhibition by antithrombin or HCII is almost the same in the presence of fibrin as it is in its absence (Figure 10).

2.1 Characteristics of Medium Molecular Weight Heparin:

Because the chains of MMWH are of sufficient length to bridge antithrombin to thrombin, the anti-factor IIa (*i.e.*, the ability of MMWH to catalyze or activate factor IIa (thrombin) inhibition by antithrombin) is the same as its anti-factor Xa activity (*i.e.*, the ability to catalyze factor Xa inhibition by antithrombin). In contrast, LMWH has greater anti-factor Xa activity than anti-factor IIa activity because more than half of the chains of LMWH are too short to bridge antithrombin to thrombin. Although unfractionated heparin also has equivalent anti-factor Xa and anti-factor IIa activity, it differs from the MMWH compositions of the invention in that it cannot catalyze thrombin inactivation in the presence of fibrin because the chains of unfractionated heparin are long enough to not only bridge antithrombin to thrombin, but also to bridge thrombin to fibrin.

In its typical configuration, the specific activity of the MMWH compositions of the invention is similar to that of unfractionated heparin. Thus, its anti-factor Xa and anti-factor IIa activity may range from 90 to 150 U/mg and 40 to 100 U/mg, respectively. LMWH typically has a specific anti-factor Xa activity of 100 U/mg, whereas its anti-factor IIa activity ranges from 20 to 50 U/mg, depending on the molecular weight profile of the particular LMWH preparation.

Example 3

Comparison of the Efficacy and Safety of the MMWH compositions of the Present Invention with Other Known Anticoagulants

This example illustrates a study comparing the efficacy and safety of a MMWH composition of the present invention, which is denoted in the figures as V21, LMWH, heparin and hirudin in a the rabbit arterial thrombosis prevention model. The results indicate that the MMWH compositions of the present invention are more effective than LMWH and heparin and safer than hirudin. The arterial thrombosis prevention model was modified so that both efficacy and safety could be assessed in the same animal. Efficacy was assessed by measuring flow over 90 minutes distal to a 95% stenosis in an injured rabbit aorta, and safety was assessed by measuring blood loss over 30 minutes using the rabbit ear model. The four compounds were compared at three dosage levels. Each compound was administered as a bolus and infusion for 90 minutes. The doses listed in the following figures represent the bolus and infusion/60 minutes, administered for 90 minutes. The doses for heparin are shown as units/Kg, for LMWH and V21 as mg/Kg and for hirudin as mg/Kg. V21 has similar anti-Xa activity to LMWH and about twice the anti-IIa activity of LMWH. Thus, the specific activity of LMWH is 100 anti-Xa units/mg and 30 anti-IIa units/mg. The specific activity of V21 is 100 anti-Xa units/mg and 60 anti-IIa units/mg, whereas the specific activity of heparin is about 150 anti-Xa units and 150 anti-IIa units/mg. The anticoagulants were compared in the following dosages. Heparin 50 units/Kg and 75 units/Kg; LMWH and V21 0.5, 1.0 and 1.5 mg/Kg; Hirudin 0.1/0.1, 0.1/0.2 and 0.1/0.3 mg/Kg.

For comparative purposes, 50 units of heparin is equivalent to 0.5 mg of LMWH or V21 in terms of anti-Xa activity, but has more than twice the anti-IIa activity of 0.5 mg of V21 and about 4 times the anti-IIa activity of LMWH. For equivalent anti-Xa activity, V21 has about twice the anti-IIa activity of LMWH.

The results obtained during this study are set forth in Figures 11, 12 and 13. Figure 11 compares the efficacy of the four anticoagulants using cumulative time that the aorta remained patent over the 90 minutes of observation as the outcome measure of efficacy. One hundred percent accumulated patency reflects complete patency and 0% cumulative patency reflects immediate and sustained thrombotic occlusion. The stenosed aorta clotted immediately and remained occluded for the full 90 minutes in the control animals, in the rabbits treated with low dose heparin (50/50 unit/Kg) and low dose LMWH (0.5/0.5 mg/Kg). There was a dose response with all four anticoagulants. However, the model was resistant to the antithrombotic effects of heparin and LMWH. Thus, both heparin in a dose of 75/75 units/Kg and LMWH in a dose of 1.0 mg/1.0 mg/Kg were ineffective (percent cumulative patency of 14% and 2% respectively), and LMWH 1.5/1.5 mg/Kg showed only limited effectiveness (38% cumulative patency). In contrast, the model was very responsive to the antithrombotic effects of V21 and hirudin. Thus, V21 at a dose of 0.5/0.5

mg/Kg was more effective than heparin at a dose of 75/75 units/Kg and more effective than LMWH in doses of 1.0/1.0 mg/Kg and 1.5/1.5 mg/Kg. Thus, V21 was at least three fold more potent than LMWH.

Figure 12 illustrates the effects of the four anticoagulants on 30 minute blood loss. A dose response was observed with LMWH, V21, and hirudin. At doses that showed greater efficacy, V21 was much safer than LMWH, and at doses that showed equivalent efficacy, V21 was safer than hirudin. V21 was also much more effective than heparin at doses that produced a similar degree of blood loss.

The comparative safety and efficacy of V21 and LMWH is illustrated in Figure 13. Based on the data (*i.e.*, three animals in each group), V21 appears to about 4 times more potent than LMWH on a weight basis. Therefore, for equivalent anti-Xa activity, V21 is 4 times more potent than LMWH, and for equivalent anti-IIa activity, V21 is about twice as potent. Such data support the importance of fibrin-bound thrombin in promoting thrombogenesis, since V21 is more effective against fibrin-bound thrombin than LMWH or heparin. At doses of 0.5 mg/Kg and 1.0 mg/Kg, V21 appears to be as safe as LMWH (although it is much more effective), but at a dose of 1.5 mg/Kg, LMWH produced much more bleeding than V21. Thus, V21 appears to have a more favorable efficacy to safety profile than LMWH.

Example 4

Studies Comparing the MMWH compositions of the Present Invention (V21) with LMWH

The efficacy of V2l (lot # D32) has been compared with LMWH (Enoxaparin) in both a heparin-sensitive and heparin-resistant thrombosis model in rabbits. The heparin-sensitive model is a venous thrombosis prophylaxis model and the heparin-resistant model is a venous thrombosis treatment model. V21 and LMWH have similar effects ex-vivo on the anti-factor Xa level and on the APTT (Figures 14 and 15). Therefore, the two anticoagulants were compared on a gravimetric basis.

1.1 Prophylaxis Model

a. Method: Twenty seven male New Zealand White rabbits weighing between three and four kilograms are randomized into 3 treatment groups.

b. Anaesthesia: Anaesthesia is induced by a mixture of intramuscular ketamin (50 mg/kg) and xylazine (2 mg/kg) and maintained by isoflurane (1-3 %) and oxygen (1L/min).

c. Surgical Procedure: The ventral cervical area is shaved and two 22-gauge catheters (Becton-Dickinson, Sandy, UT) are inserted into the left central auricular artery and the marginal auricular vein for blood sampling and for intravenous administration of treatments. The right facial and external jugular vein are exposed through the ventral cervical skin incision. A 2 cm segment of the jugular vein is isolated from surrounding tissues and side branches are ligated using 4-0 silk suture. At this time control arterial blood sample is collected (1.8 ml of blood into 0.2 ml of 3.6% sodium citrate). Blood samples are spun and plasma stored in

-70°C for blood coagulation studies (A.PTT, TCT, and anti-Xa). Intravenous bolus of I-125 labelled rabbit fibrinogen (10 µL, ~ 1.000.000.00 CPM) is administered. Thereafter rabbits are randomized to one of the following iv treatments:

1. Saline (n=9) iv bolus of Iml of saline

2. Low molecular weight heparin (n=9)(Lovenox, enoxaparin sodium, lot # 923, Rhone-Poulenc Rorer, Montreal, Quebec, Canada) in a dose of 0.50 mg/kg (n3), 1.00 mg/kg (n3), 1.5 mg/kg (n=3).

3. V-21 (n=9) (D-32, lot # 521982132) in a dose of 0.50 mg/kg (n=3), 1.00 mg/kg (n=3), and 1.5, mg/kg (n=3).

Four minutes after the treatment administration the right jugular vein is damaged in the length of 2 cm by 15 passages of the inflated balloon catheter (#4, Fogarty thrombectomy catheter). The balloon catheter is introduced into the right jugular vein via the right facial vein. Right after balloon vein injury, the catheter is withdrawn and a second arterial blood sample is taken. In addition, 1 ml of blood is also collected to measure radioactivity. Blood stasis is then induced within the 2 cm right jugular vein segment by placing two tourniquets around the vein. After 15 minute occlusion the jugular vein segment is excised and opened onto a pre-weighed square and weighed. Thereafter, the third arterial blood sample is collected for blood coagulation assay analysis.

d. End-points:

Clot weight (%) is calculated as a percentage of blood by weight trapped in the venous segment. Clot radioactivity (%) is calculated as a percentage of 1 ml of whole blood radioactivity. Plasma samples were analyzed for APTT, TCT and anti-Xa.

Schematic diagram of the procedure is shown in appended Figure 16.

e. Results:

As shown in Figures 16, 17 and 18 (for clot weight) and Figures 19, 20 and 21 (clot radioactivity), both agents are effective in this heparin-sensitive model, but V21 produces a steeper dose response and in more effective than LMWH at the two higher doses.

1.2 Treatment of Deep Vein Thrombosis in Rabbit Model

a. 24 Hour Follow-up

The purpose of this study was to compare the efficacy of V-21 with LMWH both administered subcutaneously in the rabbit model of deep vein thrombosis.

Twenty four specific pathogen free, New Zealand White, male rabbits (3-4 kg of b. wt.) were anesthetized by intramuscular injection of ketamin (50 mg/kg) and xylazine (2 mg/kg). The ventral cervical area was shaved and prepped with alcohol and iodine solution. Venous and arterial catheters were inserted into the left central auricular artery and the marginal auricular vein using an 22 gauge intravenous catheter (Angiocath, Becton Dickinson Vascular Access, Sandy, Utah., USA) for blood sample collection, and for intravenous administration of fluids and anticoagulants. Rabbits were transferred into an operating room and maintained on inhalation anesthesia which consisted of a mixture of isoflurane (1-4%), oxygen (1 L/min) and nitrous oxide (0.5 L/min) delivered by a face mask.

b. Clot Formation: The right external jugular and the facial vein were exposed through the ventral cervical skin incision. Segmental occlusion of the facial vein was achieved by two No 4-0 silk sutured placed 0.5 centimetres apart. All side branches of the jugular vein were ligated in the length of 4 centimetres. Fogerty thrombectomy catheter (#4 Fr) was introduced into the jugular vein via the facial vein and inflated. Four centimetres of the jugular vein was damaged by 15 passages of inflated balloon catheter and then the

catheter was withdrawn. A 1.5 centimetres occluded jugular vein segment was created using two 4-0 silk sutures placed around the damaged vein and then emptied using finger compression. One millilitre of arterial blood was drawn from the central auricular artery into the 1 ml syringe and mixed in a sterile tube with approximately 1 μCi of iodine-125 labelled rabbit fibrinogen. 0.6 millilitres of the radiolabelled blood was then drawn from the tube into a 1 ml syringe and the first 0.4 ml of labelled blood was equally divided into two tubes and left to clot. The remaining 0.2 ml was then injected into the occluded jugular vein segment via the home made cannula (23 gauge needle connected to PB #60). Clots generated in the test tubes served as baseline values for clot weight and radioactivity. Pilot studies have shown that there was around 5 % difference in clot weight or radioactivity between the clots generated in tubes and in the jugular vein. The thrombus generated in the jugular vein was left to mature for 30 minutes and the facial vein was ligated. Twenty five minutes into the thrombus maturation rabbits were randomized to receive:

1) saline treatment (n=4) 1 ml of sterile saline BID sc;
2) low molecular weight heparin (Enoxaparin sodium, Lovenox lot #923, Rhone-Poulenc Rorer, Montreal, Quebec) at a dose of 1 mg/kg BID, sc (n=4), and 3 mg/kg BID sc (n=4); and
3) V-21 (D-32, lot # 521982132) at a dose of 1 mg/kg BID sc (n=4) and at 3 mg/kg BID sc (n=4).

Thirty percent of the first dose was administered intravenously and 70 % subcutaneously; the second dose was given only subcutaneously. Just prior to tourniquet removal at 30 minutes, the thrombus was fixed to the vein wall by two silk sutures to prevent its migration in the post-operative period. There was no residual stenosis of the jugular vein left after tourniquet removal. The cervical incision was closed in a routine manner. Rabbits were left to recover breathing 100% oxygen and then transferred to the recovery room. All rabbits were euthanized at the 24 hour time interval.

c. Blood collection: Arterial blood was collected prior to surgery (control) and at 5 minutes, 1, 3, 6, 9, 12, and 24 hours alter clot maturation. At each time interval 2 millilitres of citrated blood was collected (9:1 ratio, 3.8% sodium citrate) for APTT, TCT and Xa assays. Blood loss was replaced by iv administration of saline.

d. End-points: Using thrombus weight in milligrams (AG Balances #104, Mettler Toledo, Fisher Scientific Limited, Whitby, Ontario) and thrombus radioactivity (CPM) at the time of clot induction (clot created in tubes) and at 24 hours the following end-points were calculated:

Percentage change in clot weight (PCCW) was calculated using clot weight at 24 hours minus clot weight generated in a test tube at the time of surgery divided by clot weight generated in a test tube times 100.

Clot accretion (CA) at % was calculated as follows: AC=PCCW-CL

e. Results: As illustrated in Figures 23-28, V21 is more effective than LMWH in this heparin-resistant model.

Example 5

Preparation of the MMWH compositions of the Present Invention by a Limited Periodate Oxidation/Hydrolysis of Heparin

1.1 Study of Limited Periodate Oxidation/Hydrolysis of Heparin

Heparin was dissolved in deuterated water to make 10% of stock solution. Sodium periodate was dissolved in deuterated water to make 100 mM stock solution and kept at 4°C. The periodate oxidation reaction was carried out at 2.5% of heparin concentration with increasing sodium periodate concentration, 1 mM, 2.5 mM, 5 mM, 8 mM, 10 mM, and 20 mM, at room temperature for about 18 hours. The reaction was stopped by adding 50 mM of ethylene glycol and incubation for 30 minutes. Then, the reaction mixture was brought to 0.25 N NaOH and incubated at room temperature for 3 hours. After the reaction, the pH was adjusted to pH 7 by 6 N HCl. An aliquot of each reaction mixture was run on an HPLC-GPC (G2000 column, 0.5 ml/min, injection volume 20 µl) for molecular weight analysis. The molecular weight profiles of the reaction at sodium periodate concentrations of 5 mM, 8 mM, 10 mM, and 20 mM decrease in comparison to heparin with increasing sodium periodate concentration. The result indicated that the desired cleavage can be achieved using sodium periodate concentrations of between about 5 mM and about 20 mM, and at room temperature for about 18 hours. The study (not shown) indicated that the best alkaline hydrolysis can be achieved using 0.25 N NaOH, at room temperature for 3 hours. Thus, the reaction conditions used in this experiment are called "limited periodate/hydrolysis" conditions.

1.2 Preparation of MMWH compositions of the Present Invention by Limited Periodate Oxidation/Hydrolysis

100 mg of heparin was treated using the limited periodate/hydrolysis conditions, 7 mM sodium periodate, and purified by P30 gel-filtration chromatography. 30 mg of final product, *i.e.*, V21-D32, was obtained having a molecular weight ranging from about 6,000 Daltons to about 12,000 Daltons, and having a peak molecular weight of about 9,000 Daltons.

Example 6

Studies were undertaken to select MMWH compositions with an optimal molecular weight range and to identify a manufacturing process which could be readily scaled up to obtain a heparin fraction within this range.

A molecular weight range between 6,000 and 10,000 was selected as an optimal molecular weight range. Compositions with a minimum molecular weight of 6,000, which corresponds to 20 saccharide units, should provide heparin chains that have pentasaccharide-containing chains long enough to bridge antithrombin to thrombin. With a maximum molecular weight of 10,000, which corresponds to 33 saccharide units, the chains will (a) be too short to bridge thrombin to fibrin, a phenomenon that requires chains of 40 saccharide units or more, and (b) too short to exhibit non-specific binding to plasma proteins, a phenomenon that occurs with chains of 30 saccharide units or more.

Heparin Fractions:

Unfractionated heparin was depolymerized with heparinase, nitrous acid, or periodate to yield fractions of approximately 6,000, 8,000, and 10,000 Da. While initial fractions produced by these three methods were polydispersed, more size-restricted fractions of these molecular weights were prepared using either heparinase or nitrous acid depolymerization. The characteristics of these fractions are illustrated in Table 1 along with their specific anti-Xa and anti-IIa activities.

Affinities for Antithrombin

The affinities of each of the heparin fractions for antithrombin was determined as previously described (Weitz et al, Ciruculation 1999:99:682-689). Briefly, a 1 X 1 cm. quartz cuvette containing 100 nM antithrombin in 2 ml of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) was excited at 200 nm (6-nm slit width) and intrinsic fluorescence was continuously monitored in time drive at 340 nm (6-nm slit width) with a Perkin-Elmer LS50B luminescence spectrometer. The contents of the cuvette were stirred with a micro-stir bar and maintained at 25°C with a recirculating water bath. Intrinsic fluorescence intensity was measured before (I_o) and after (I) addition of 5 to 10 ml of 10-mg/ml solutions of the various heparin fractions. Titrations were continued until there was no change in I. After the experiment, I values were read from the time drive profile and I/I_o values were calculated and plotted versus heparin concentration. The data were then analyzed as described previously (Weits et al, supra). From this analysis, stoichiometry can be obtained which is interpreted as indicating the proportion of pentasaccharide-containing chains within each heparin fraction.

The affinities are summarized in Table 2. Also illustrated is the estimated percentage of pentasaccharide-containing chains within each fraction. Fractions prepared by either heparinase or nitrous acid depolymerization exhibit similar affinities for antithrombin. Although the 10,100 Da fraction prepared by periodate depolymerization exhibits affinity for antithrombin similar to that of heparinase or nitrous acid-derived fractions, the lower molecular weight periodate-derived fractions have lower affinities consistent with their reduced anti-IIa and anti-Xa activities (Table 1). As might be expected, regardless of the method used for depolymerization, the percentage of pentasaccharide-containing chains increases as the mean molecular weight increases.

As controls for these analyses, unfractionated heparin, high and low affinity fractions of heparin prepared by affinity chromatography using an antithrombin column, enoxaparin, and synthetic pentasaccharide also were studied. As illustrated in Table 3, the high affinity fraction of heparin and synthetic pentasaccharide exhibit the highest affinity for antithrombin. Only these two preparations have 100% pentasaccharide-containing chains.

Affinities for Thrombin:

The affinities of the polydispersed heparinase, nitrous acid, and periodate-derived heparin fractions for thrombin were measured as described above except thrombin was used in place of antithrombin (Fredenburgh, JC et al. J. Biol. Chem. 1997:272:25493-25499). As illustrated in Table 4, when affinities are expressed in µg/ml, all fractions exhibited similar affinities for thrombin.

Heparin-catalyzed rates of thrombin inhibition by antithrombin in the absence or presence of fibrin monomer:

The second order rate constants for thrombin inhibition by antithrombin were measured in the absence or presence of the various heparin fractions in concentrations ranging from 0 to 600 µg/ml. Heparin-catalyzed rates of thrombin inhibition by antithrombin were measured both in the absence or presence of 4 µM fibrin monomer. The fibrin monomer was prepared as previously described, and the data were analyzed as described elsewhere (Becker DL et al, J. Biol. Chem. 1999:274:6226-6233).

The inhibitory effect of fibrin-monomer on the rates of inhibition of thrombin by antithrombin is

shown with the heparinase (Figure 29), nitrous acid (Figure 30), and periodate-derived heparin fractions (Figure 31). The background inhibition with fibrin monomer is 6-fold as determined by measuring the inhibitory effect of fibrin monomer on the heparin-catalysed rate of factor Xa inactivation by antithrombin. (Figures 32 and 33). There is less reduction in the rate of thrombin inactivation by antithrombin with the heparinase or nitrous acid-derived heparin fractions than with unfractionated heparin. In contrast, greater inhibition with fibrin monomer is seen with the periodate-derived heparin fractions (Figure 31). With the size-restricted heparinase -derived fractions, fibrin-monomer produces no more than background inhibition.

Heparin-catalyzed inhibition of factor Xa by antithrombin:

The second order rate constants for factor Xa inhibition by antithrombin were measured in the absence or presence of the various heparin fractions in concentrations ranging from 0 to 1,500 μg/ml as described elsewhere (Becker et al, supra). The results for the heparinase, nitrous acid, and periodate-derived fractions are illustrated in Figures 34 to 36, respectively. When added in gravimetrically equivalent amounts, all of the heparin fractions produce less catalysis of factor Xa inhibition by antithrombin than unfractionated heparin.

Augmentation of thrombin binding to fibrin:

I^{125}-labeled thrombin binding to fibrin was measured in the absence or presence of the various heparin fractions in concentrations ranging from 0 to 7,500 nM as previously described (Becker et al, supra). Unfractionated heparin was used as a control in these experiments. The results with heparinase, nitrous acid, and periodate-derived heparin fractions are illustrated in Figures 37 to 39, respectively. Regardless of the method of depolymerization, the 10,000 Da fractions augment thrombin binding to fibrin to a greater extent than the lower molecular weight fractions. This is best illustrated with the more size-restricted heparinase or nitrous acid-derived fractions (Figures 40 and 41, respectively).

Antithrombotic activity of heparin fractions:

An extracorporeal circuit was used to compare the antithrombotic activity of the heparin fractions. As previously described (Weitz et al, supra), different concentrations of each of the heparin fractions was added to recalcified human whole blood spiked with ^{125}I-labeled human fibrinogen and maintained at 37°C in a water bath. A peristaltic pump was then used to circulate the blood through a 40 μ blood filter. Clotting of blood within the filter was detected by (a) measuring pressure proximal to the filter with an in-line pressure gauge, and (b) removing serial blood samples from the reservoir and counting residual radioactivity as an index of fibrinogen consumption. Starting activated clotting times also were measured.

As illustrated in Table 5, regardless of the method of depolymerization, fractions of 10,000 Da were effective at a concentration of 10 μg/ml. Thus, filter patency was maintained during the 90 min observation period and fibrinogen consumption was less than 10%. At a concentration of 10 μg/ml, the heparinase-derived 8,000 Da fraction was effective. The 6,000 Da heparinase fraction was effective at 14 μg/ml. Although patency was maintained with 14 or 16 μg/ml of the 5,600 Da nitrous acid-derived fractions, fibrinogen consumption was 33 and 20%, respectively. As a control, enoxaparin was also evaluated. This drug was ineffective at 10 or 20 μg/ml with filter failure occurring at 30 and 55 min, respectively.

The antithrombotic activities of the more size-restricted heparinase and nitrous acid-derived heparin fractions are illustrated in Table 6. All fractions were tested at a concentration of 10 μg/ml with 10 μg/ml enoxaparin serving as a control. Except for the 5,300 Da heparinase-derived fraction, all of the heparin fractions were effective in maintaining patency for > 90 min and reducing fibrinogen consumption to < 10%. In contrast, enoxaparin was ineffective with filter failure occurring at 30 min and fibrinogen consumption of 73%. The antithrombotic activities of the more size-restricted heparinase, nitrous acid-derived heparin and periodate fractions are illustrated in Table 7. All fractions were tested at a concentration of 10 μg/ml with 10 μg/ml enoxaparin serving as a control. The heparinase and nitrous acid derivative fractions were effective in aminating patency. The periodate-derived fraction and enoxaparin were less effective.

It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications and publications, are incorporated herein by reference in their entirety for all purpose.

TABLE 1

CHARACTERISTICS OF THE HEPARIN FRACTIONS PROVIDED BY LEO

Depolymerization Method	Molecular Weight	Polydispersity	Anti-IIa	Anti-Xa
Heparinase	6,000	1.5	72	106
	8,500	1.5	100	134
	10,350	1.5	152	111
$HN\ 0_2$	5,600	1.5	59	118
	8,200	1.4	100	152
	10,300	1.4	119	180
IO_4^-	6,700	1.5	11	30
	7,900	1.5	19	43
	10,100	1.5	43	88
	10,300	1.5	42	84
Heparinase	5,300	1.2	22	81
	8,450	1.2	67	116
	9,750	1.3	87	155
$HN0_2$	5,900	1.2	32	95
	7,700	1.3	84	123
	9,300	1.2	106	162

TABLE 2

AFFINITIES OF HEPARIN FRACTIONS FOR ANTITHROMBIN AND PERCENTAGE OF PENTASACCHARIDE-CONTAINING CHAINS IN EACH FRACTION

Glycosaminoglycan		K_d	Pentasaccharide-containing
		nM	%
Heparinase	6,000	91.2 ± 15.9	14.8
	8,050	61.7 ± 4.2	20.8
	10,350	48.0 ± 7.8	27.0
HNO_2	5,600	55.6 ± 0.2	16.0
	8,200	42.5 ± 9.1	25.2
	10,300	37.5 ± 2.9	31.5
IO_4^-	6,700	170.1 ± 27.4	6.8
	8,200	140.3 ± 4.5	10.5
	10,300	57.0 ± 28.3	25.6
Heparinase	5,300	421.6 ± 72.3	15.9
	8,450	167.0 ± 17.0	29.4
	9,750	138.4 ± 2.2	32.2
HNO_2	5,900	32.8 ± 0.3	21.1
	7,700	23.1 ± 4.1	26.9
	9,300	17.0 ± 0.3	36.6

TABLE 3

AFFINITIES OF UNFRACTIONATED HEPARIN, HEPARIN WITH HIGH OR LOW AFFINITY FOR ANTITHROMBIN, ENOXAPARIN AND SYNTHETIC PENTASACCHARIDE FOR ANTITHROMBIN AND PERCENTAGE OF PENTASACCHARIDE-CONTAINING CHAINS IN EACH PREPARATION

Glycosaminoglycan	K_d	Pentasaccharide-containing
	nM	%
Unfractionated heparin	31.7	43.1
High affinity heparin	10.7	114
Low affinity heparin	6670.0	1.0
Enoxaparin	46.8	14.4
Pentasaccharide	31.0	102

TABLE 4

AFFINITIES OF HEPARINASE AND NITROUS ACID-DERIVED HEPARIN FRACTIONS FOR THROMBIN

Glycosaminoglycans		K_d nM	K_d µg/ml
Heparinase	6,000	1517 ± 196	9.1 ± 1.2
	8,050	872 ± 9	7.0 ± 0.1
	10,350	699 ± 97	7.2 ± 1.0
HNO_2	5,600	1288 ± 92	7.2 ± 0.5
	8,200	695 ± 37	5.7 ± 0.3
	10,300	632 ± 51	6.5 ± 0.5
IO_4	6,700	731 ± 159	4.9 ± 1.1
	7,900	587 ± 8	4.6 ± 0.1
	10,100	285 ± 5	2.9 ± 0.5

TABLE 5

ANTITHROMBOTIC ACTIVITY OF HEPARINASE, NITROUS ACID, AND PERIODATE-DERIVED HEPARIN FRACTIONS AND ENOXAPARIN IN AN EXTRACORPOREAL CIRCUIT

Glycosaminoglycan		Concentration	Time to Filter Failure	Fibrinogen Consumption	Starting ACT
		µg/ml	min	%	sec
Heparinase	6 kDa	8	75	82	271
		10	>90	68	248
		12	>90	31	226
		14	>90	7	335
	8 kDa	5	40	70	241
		6	45	70	230
		8	>90	54	256
		10	>90	6	282
	10 kDa	5	45	72	231
		6	>90	36	299
		8	>90	29	300
		10	>90	4	327
HNO_2	5.6 kDa	10	>90	29	238
		12	>90	57	235
		14	>90	33	238
		16	>90	20	264
	8.2 kDa	10	60	81	239
		11	>90	28	313
		12	>90	10	301
		16	>90	8	428
	10.3 kDa	8	>90	14	303
		10	>90	28	287
		11	>90	7	341
		12	>90	7	359
IO_4^-	6.7 kDa	10	45	68	–
	7.9 kDa	10	90	73	299
	10.1 kDa	10	>90	6	318
Enoxaparin		10	30	73	202
		20	55	64	231

TABLE 6

COMPARISON OF ACTIVITY OF 10 μg/ml HEPARINASE, NITROUS ACID- DERIVED HEPARIN FRACTIONS WITH ENOXAPARIN IN EXTRACORPOREAL CIRCUIT

Glycosaminoglycan		Time to Filter Failure	Fibrinogen Consumption	Starting ACT
		min	%	sec
Heparinase	5,300	30	80	205
	8,450	>90	9	283
	9,750	>90	8	312
HNO_2	5,900	>90	11	278
	7,700	>90	5	314
	9,300	>90	8	557
Enoxaparin		30	73	202

TABLE 7

COMPARISON OF ACTIVITY OF 10 µg/ml HEPARINASE, NITROUS ACID-DERIVED AND PERIODATE-DERIVED HEPARIN FRACTIONS WITH ENOXAPARIN IN EXTRACORPOREAL CIRCUIT

Glycosaminoglycan		Time to Filter Failure	Fibrinogen Consumption	Starting ACT
		min	%	sec
Heparinase	8,450	>90	7.1	289
HNO_2	7,700	>90	5.6	296
IO4	7,900	30	77	242
Enoxaparin		30	80	232

WE CLAIM:

1. A medium molecular weight heparin (MMWH) composition comprising a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons.
2. The MMWH composition in accordance with claim 1, wherein said MMWH composition (1) inhibits fibrin-bound thrombin and fluid-phase thrombin by catalyzing antithrombin, and (2) inhibits thrombin generation by catalyzing factor Xa inactivation by antithrombin.
3. The MMWH composition in accordance with claim 1, wherein said MMWH composition has an anti-factor IIa activity of about 40 U/mg to about 100 U/mg, and an anti-factor Xa activity of about 90 U/mg to about 150 U/m
4. The MMWH composition in accordance with claim 3, wherein said MMWH composition has an anti-factor IIa activity of about 60 U/mg to about 75 U/mg, and an anti-factor Xa activity of about 100 U/mg to about 125 U/mg.
5. The MMWH composition in accordance with claim 4, wherein said MMWH composition has an anti-factor IIa activity of about 65 U/mg, and an anti-factor Xa activity of about 115 U/mg
6. The MMWH composition in accordance with claim 1, wherein said MMWH composition comprises a mixture of sulfated oligosaccharides having molecular weights ranging from about 8,000 Daltons to about 10,000 Daltons.
7. The MMWH composition in accordance with claim 1, wherein said MMWH composition has an average molecular weight of about 9,000.
8. The MMWH composition in accordance with claim 1, wherein at least 31% of said sulfated oligosaccharides have a molecular weight greater than or equal to about 7,800.
9. The MMWH composition in accordance with claim 1, wherein at least 25% of said sulfated oligosaccharides have a molecular weight greater than or equal to about 10,000 Daltons.
10. A medium molecular weight heparin (MMWH) composition comprising a mixture of oligosaccharides derived from heparin characterized by one or more of the following characteristics:
    (a) having antithrombin- and heparin cofactor II (HCII)-related anticoagulant activity *in vitro;*
    (b) the oligosaccharides are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin or HCII to thrombin;
    (c) having at least 15%, 20%, 25%, 30%, 35%, or 40% oligosaccharides with at least one or more pentasaccharide sequence;
    (d) enriched for oligosaccharides having a molecular weight range from about 6,000 to about 11,000; 7,000 to 10,000; 7,500 to 10,000; 7,800 to 10,000; 7,800 to 9,800; or 7,800 to 9,600; 8,000 to 9,600;
    (e) the oligosaccharides have a mean molecular weight of about 7,800 to 10,000, preferably 7,800 to 9,800, more preferably 8,000 to 9,800;
    (f) at least 30%, 35%, 40%, 45%, or 50% of the oligosaccharides have a molecular weight greater than or equal to 6000 Daltons, preferably greater than or equal to 8000 Daltons;
    (g) a polydispersity of 1.1 to 1.5, preferably 1.2 to 1.4, most preferably 1.3;

(h) having similar anti-factor Xa and anti-factor IIa activities, preferably a ratio of anti-factor Xa activity to anti-factor IIa activity from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1;

(i) an anti-factor Xa activity from about 80 IU/mg to about 155 IU/mg, preferably 90 IU/mg to about 130 IU/mg, more preferably, from about 95 IU/mg to about 120 IU/mg and, most preferably 100-110 IU/mg; and

(j) an anti-factor IIa activity from about 20 IU/mg to about 150 IU/mg; preferably 40 IU/mg to about 100 IU/mg, more preferably, from about 80 IU/mg to about 100 IU/mg, most preferably about 90-100 IU/mg.

11. A MMWH composition in accordance with claim 10 which has the characteristics of (a), (b), (c) and (d); (a) (b), (c), and (e); (b), (c), (e), and (g); (b), (d), (c), (e), and (h); (b) (c), (d), and (g); (b), (e), (g), (i), and (j); (b), (e), (f), (g), (i) and (j); or (a) through (j).

12. A MMWH composition in accordance with claim 10 enriched for oligosaccharides having a molecular weight range of 7,800 to 8,800, preferably 7,800 to 8,600, more preferably 7,800 to 8,500, most preferably 8,000 to 8,500.

13. A MMWH composition in accordance with claim 10 enriched for oligosaccharides having a molecular weight range of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most preferably 9,400 to 9,600.

14. A MMWH composition in accordance with claim 10 comprising oligosaccharides having a mean molecular weight of 7,800 to 8,800, preferably 7,800 to 8,600, more preferably 7,800 to 8,500, most preferably 8,000 to 8,500.

15. A MMWH composition in accordance with claim 10 comprising oligosaccharides having a mean molecular weight of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most preferably 9,400 to 9,600.

16. A MMWH composition as claimed in claim 10, 11, 12, 13, 14, or 15 derived from heparinase depolymerization or nitrous acid depolymerization of unfractionated heparin.

17. A method for treating a thrombotic condition in a subject comprising administering to the subject a pharmacologically acceptable dose of a medium molecular weight heparin (MMWH) composition as claimed in any of the preceding claims.

18. The method in accordance with claim 17, wherein said thrombotic condition is arterial thrombosis, coronary artery thrombosis, venous thrombosis, or pulmonary embolism.

19. The method in accordance with claim 17, wherein said MMWH composition is administered by injection.

20. A method of preventing the formation of a thrombus in a subject at risk of developing thrombosis comprising administering to the subject a pharmacologically acceptable dose of a medium molecular weight heparin (MMWH) composition as claimed in any of the preceding claims.

21. The method in accordance with claim 20, wherein the subject is at increased risk of developing thrombosis due to a medical condition which disrupts hemostasis.

22. The method in accordance with claim 21, wherein the medical condition is coronary artery disease, or atherosclerosis.

23. The method in accordance with claim 20. wherein the subject is at increased risk of developing thrombosis due to a medical procedure.

24. The method in accordance with claim 23, wherein the medical procedure is cardiac surgery, cardiopulmonary bypass, catheterization, or atherectomy.

25. The method in accordance with claim 24, wherein the catheterization is cardiac catheterization

26. A method for inhibiting thrombus formation in a patient comprising the step of administering to the patient a pharmacologically acceptable dose of a medium molecular weight heparin (MMWH) composition as claimed in any of the preceding claims.

27. A pharmaceutical composition comprising a MMWH composition as claimed in any of the preceding claims and a pharmaceutically acceptable carrier.

28. A method for treating deep vein thrombosis in a patient comprising administering to a patient undergoing orthopedic surgery a therapeutically effective amount of a MMWH composition as claimed in any of the preceding claims.

29. A method for preventing a pulmonary embolism in a subject comprising administering to the subject a therapeutically effective amount of a MMWH composition as claimed in any of the preceding claims.

30. A method for preparing a medium molecular weight heparin (MMWH) composition comprising:

(a) subjecting unfractionated heparin to a limited periodate oxidation reaction such that only the iduronic acids of the unfractionated heparin are oxidized;

(b) subjecting the oxidized unfractionated heparin of step (a) to alkaline hydrolysis; and

(c) recovering said MMWH composition, wherein the MMWH composition comprises a mixture of sulfated oligosaccharides having molecular weights ranging from about 8,000 Daltons to about 12,000 Daltons.

31. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for treating a thrombotic condition, or preventing the formation of a thrombus in a subject at risk of developing thrombosis.

32. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for inhibiting fibrin-bound thrombin and thrombin generation in a subject.

33. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for treating deep vein thrombosis.

34. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for preventing pulmonary embolism in a subject.

(B) EFFECT OF HEPARIN ON THE AFFINITY OF α-THROMBIN FOR FIBRIN

HEPARIN (μM)	Kd (μM)
0	3.22
0.1	0.25
0.25	0.15
1	0.79
20	4.26

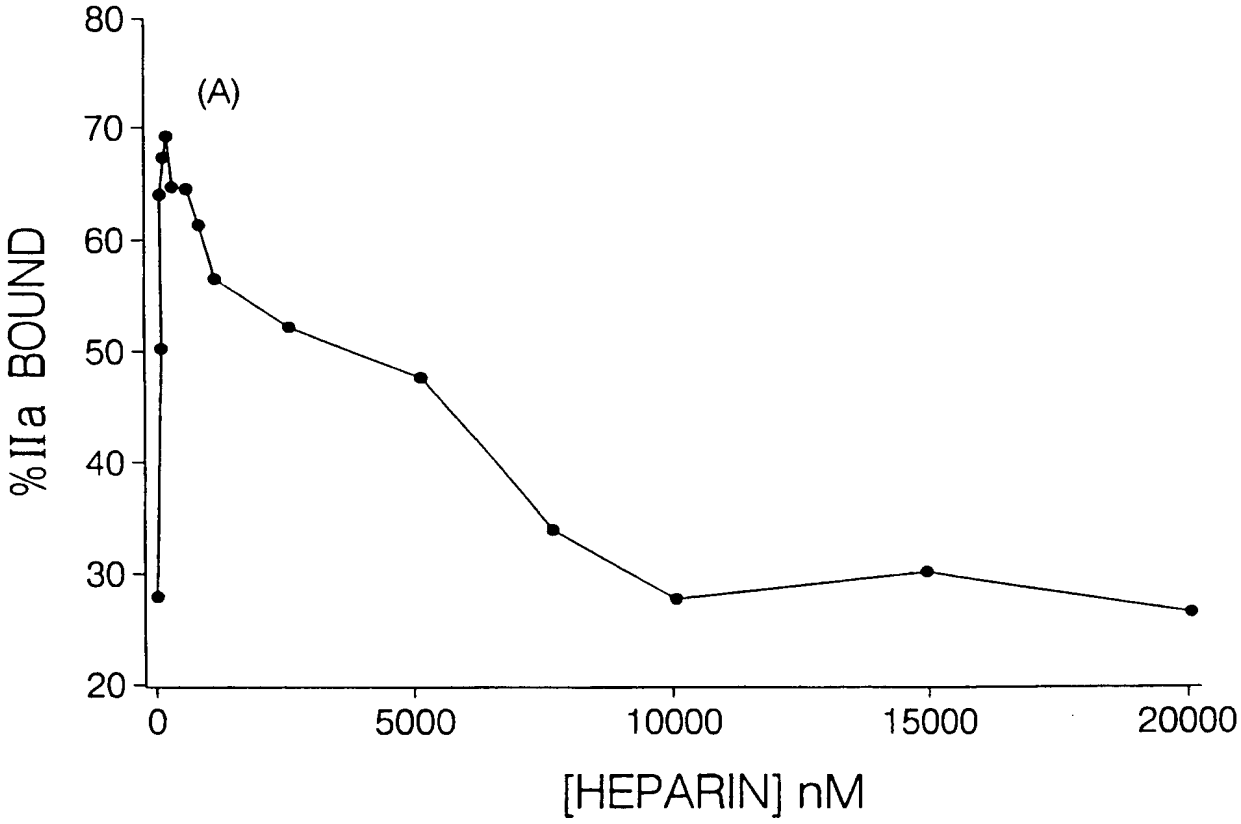


Fig. 1

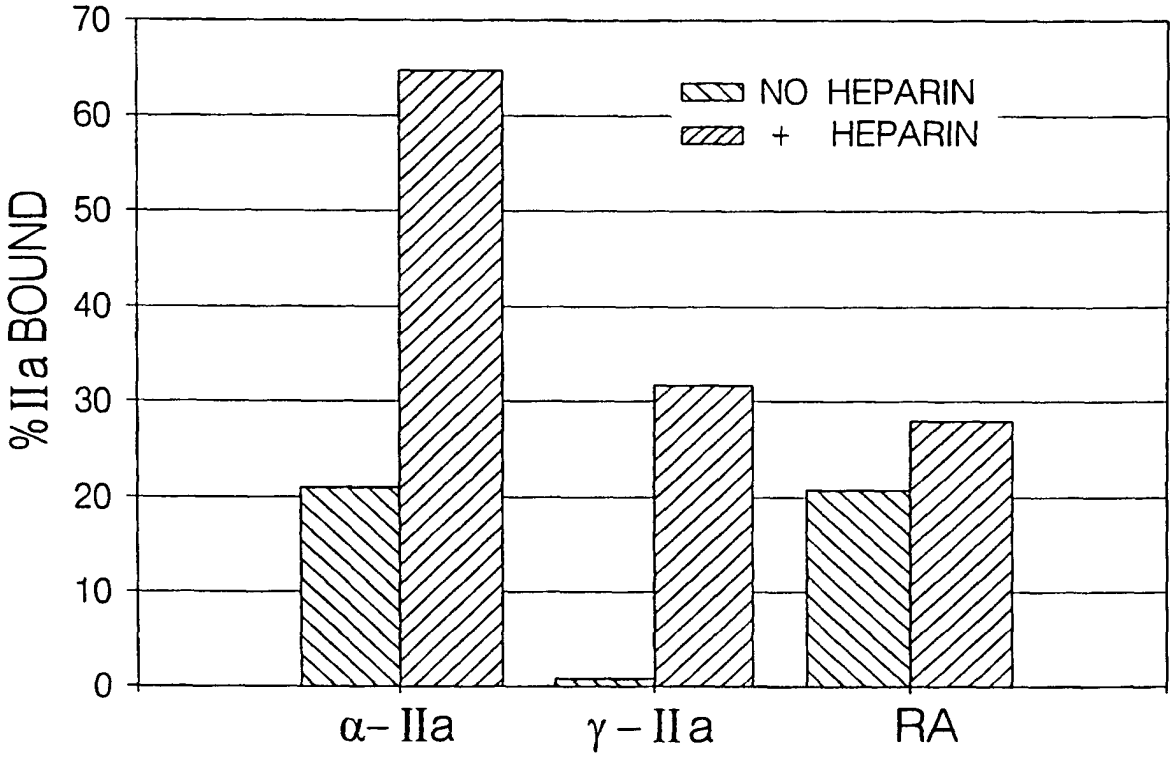


Fig. 2

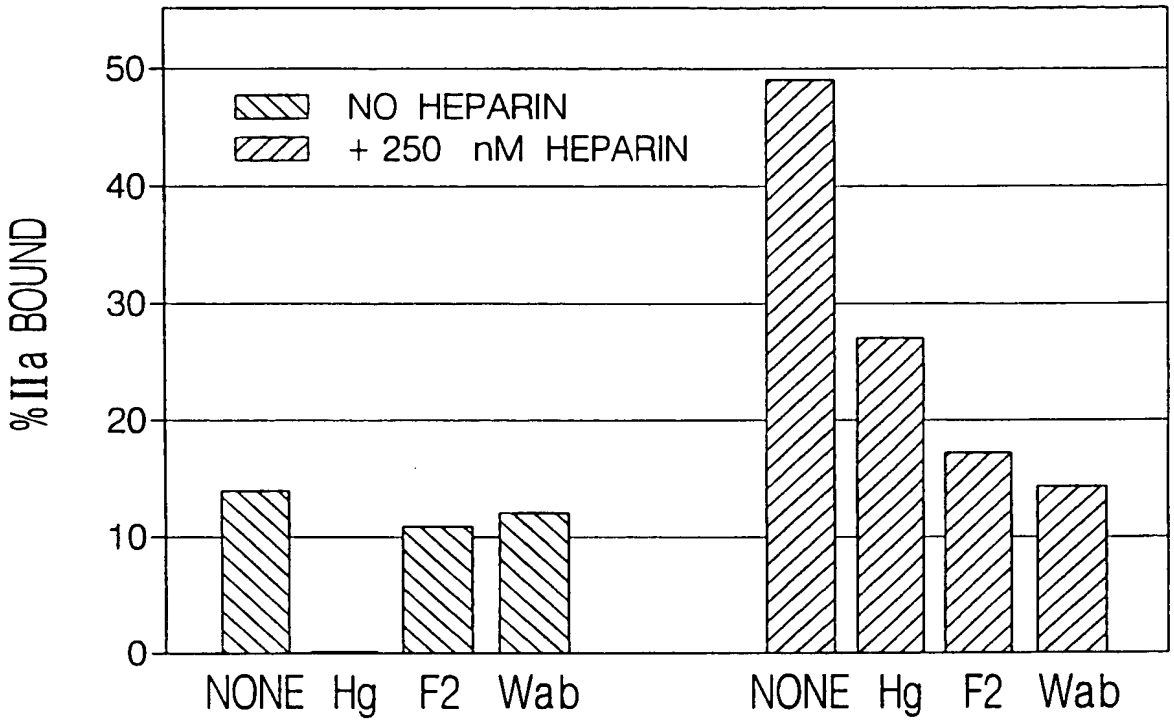


Fig. 3

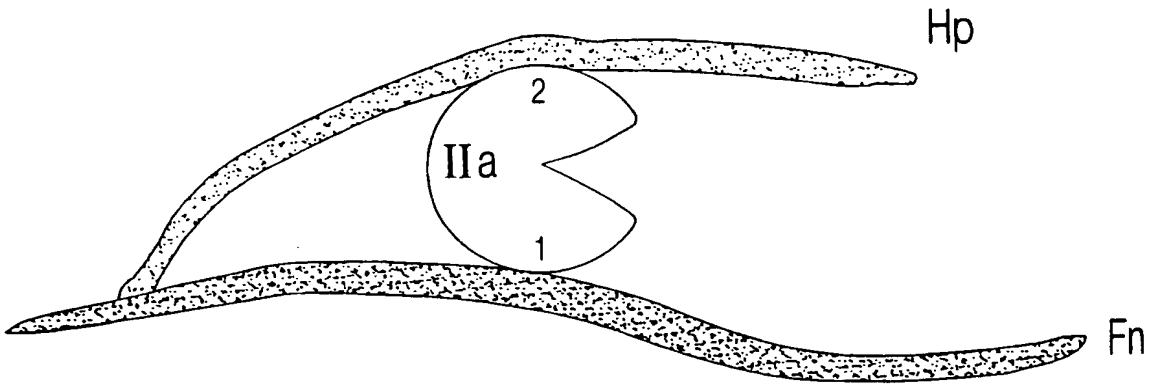


Fig. 4

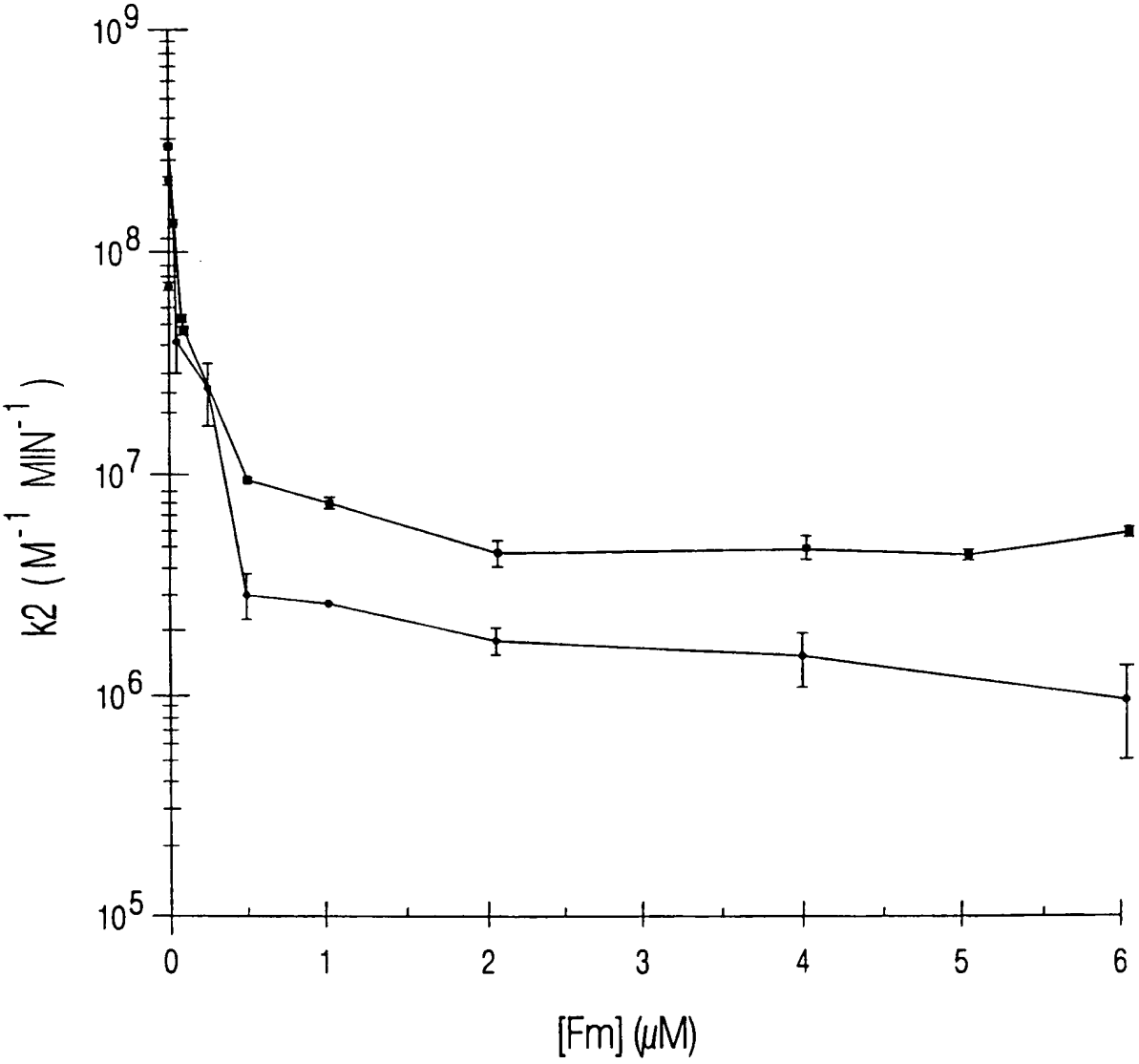


Fig. 5

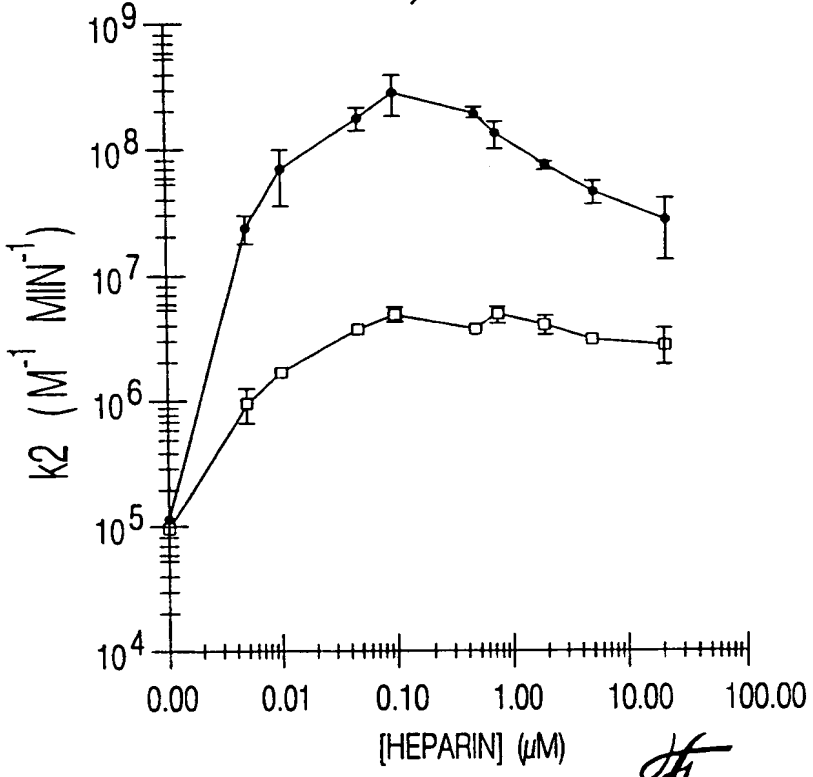


Fig. 6A

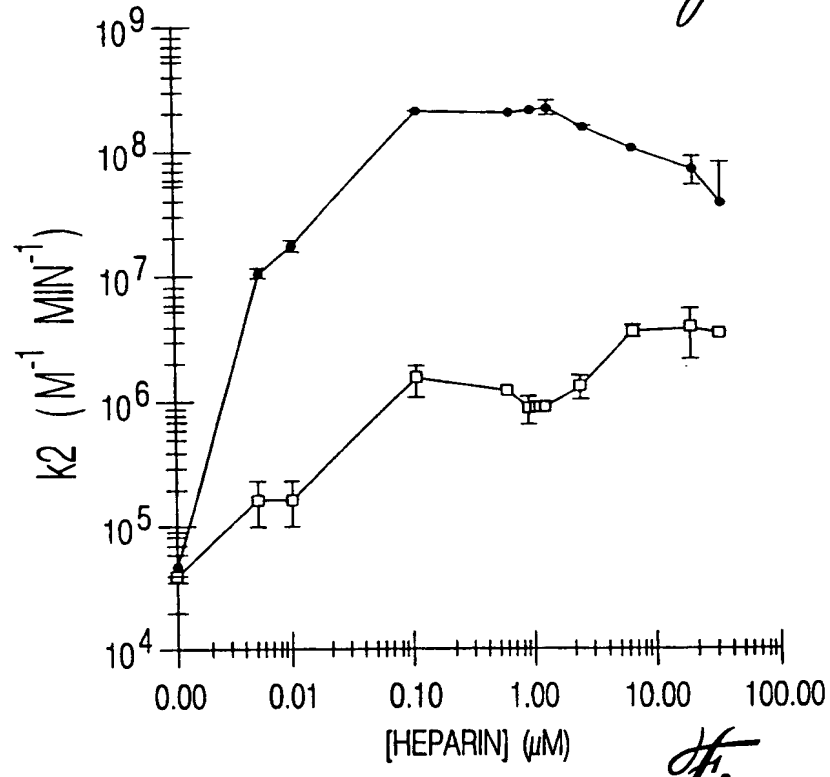


Fig. 6B

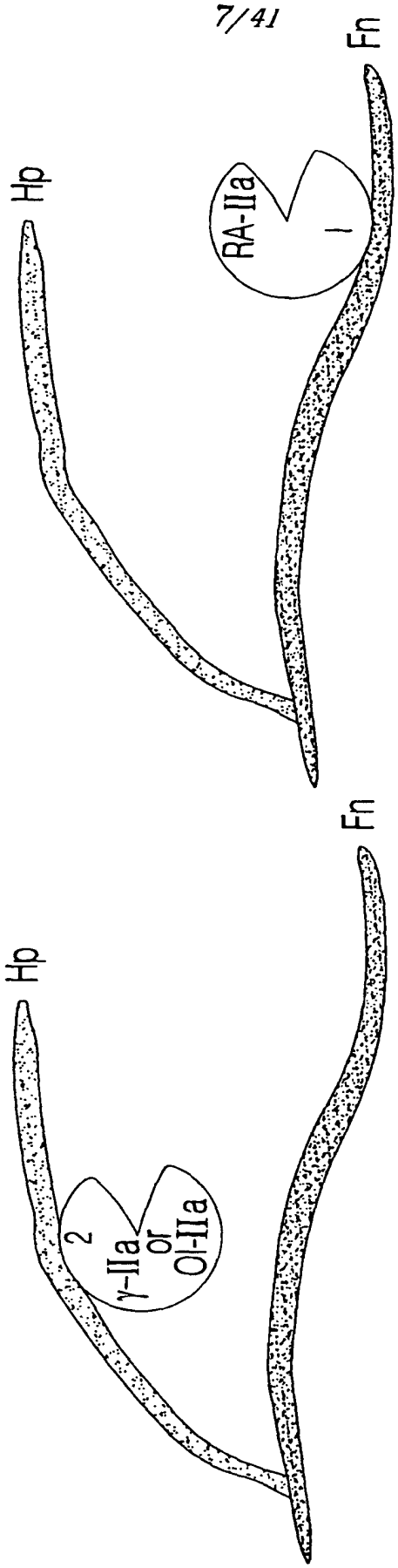


Fig. 7

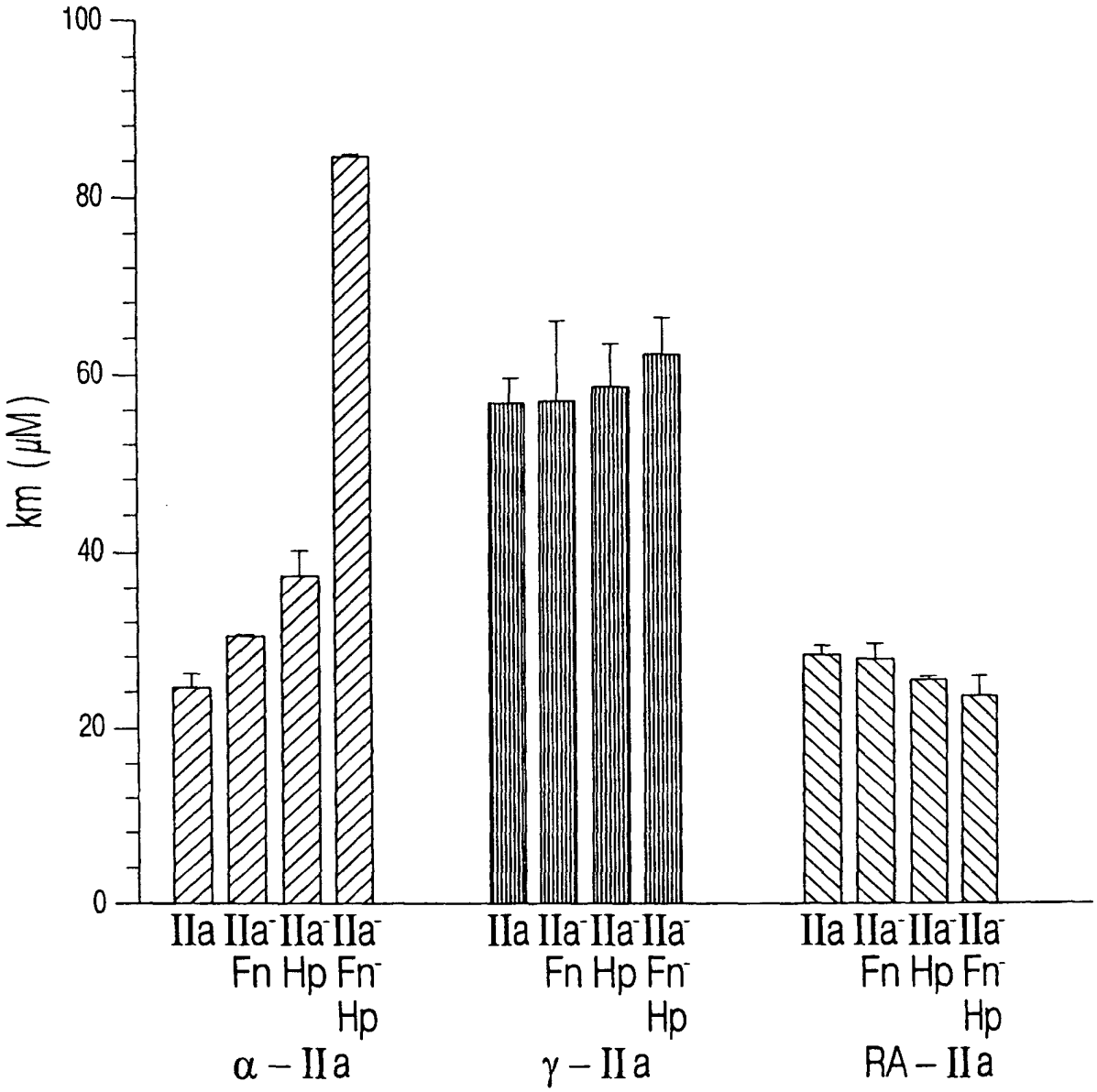


Fig. 8

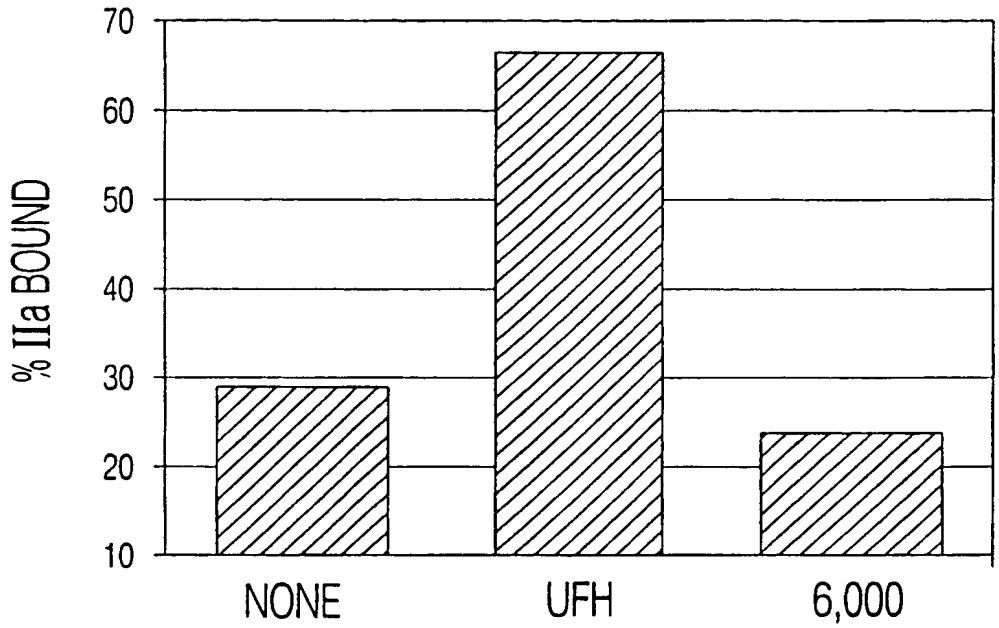


Fig. 9

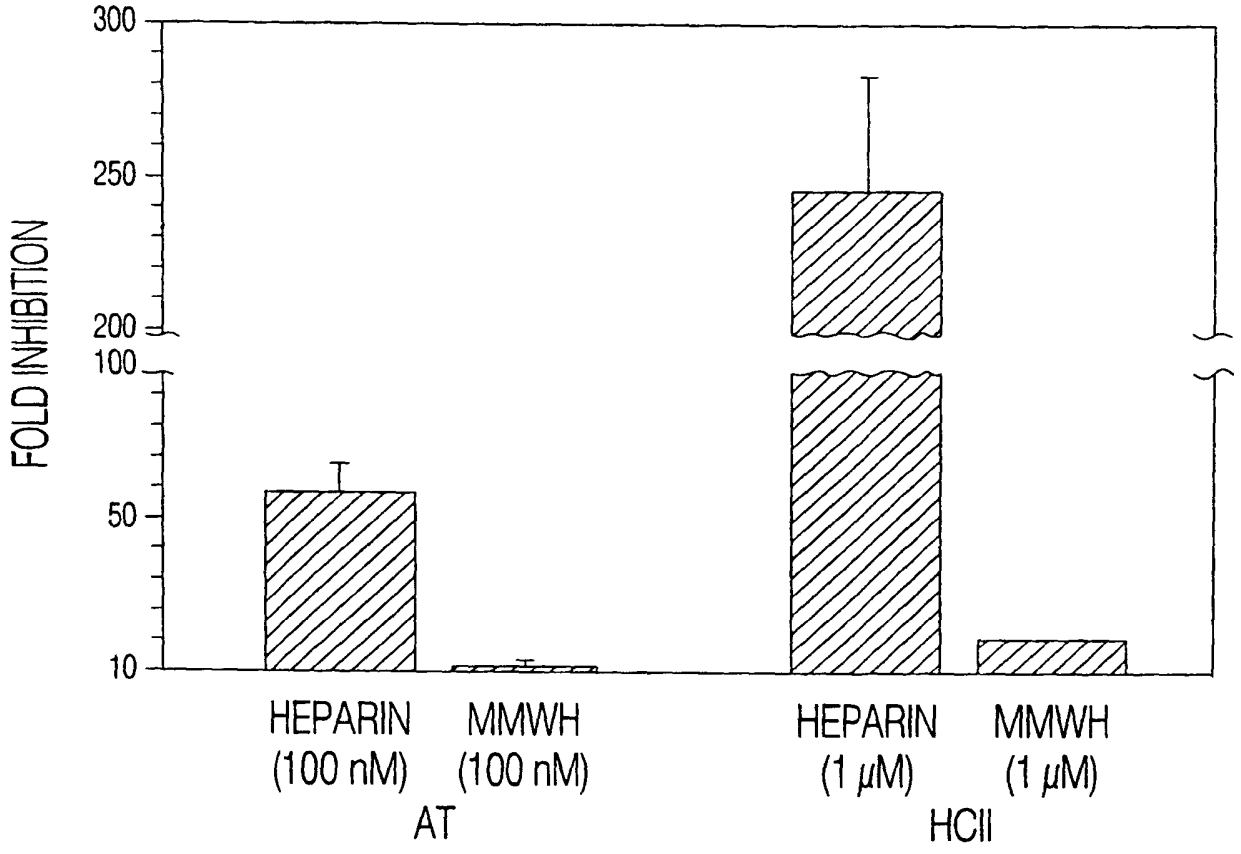


Fig. 10

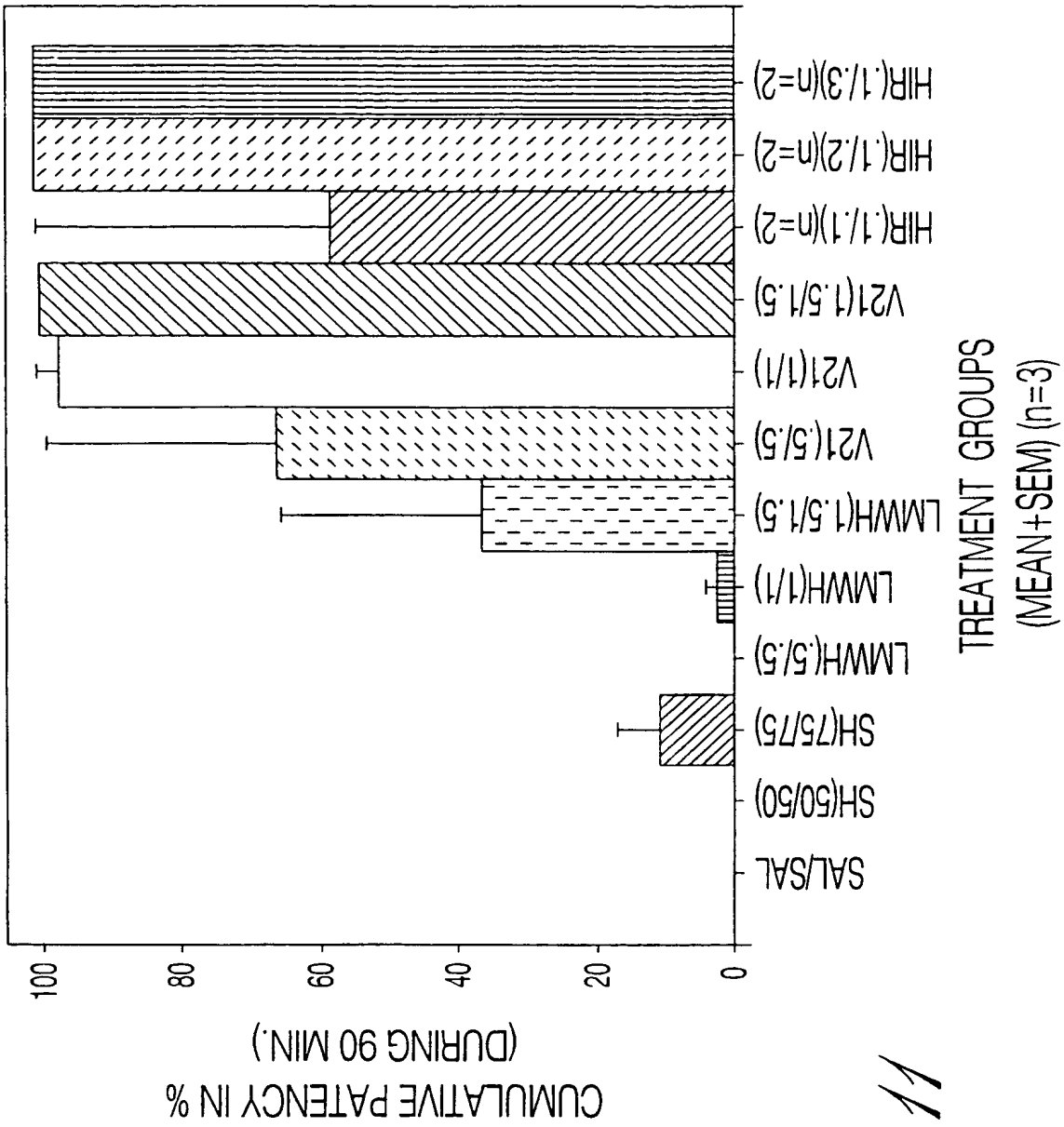


Fig. 11

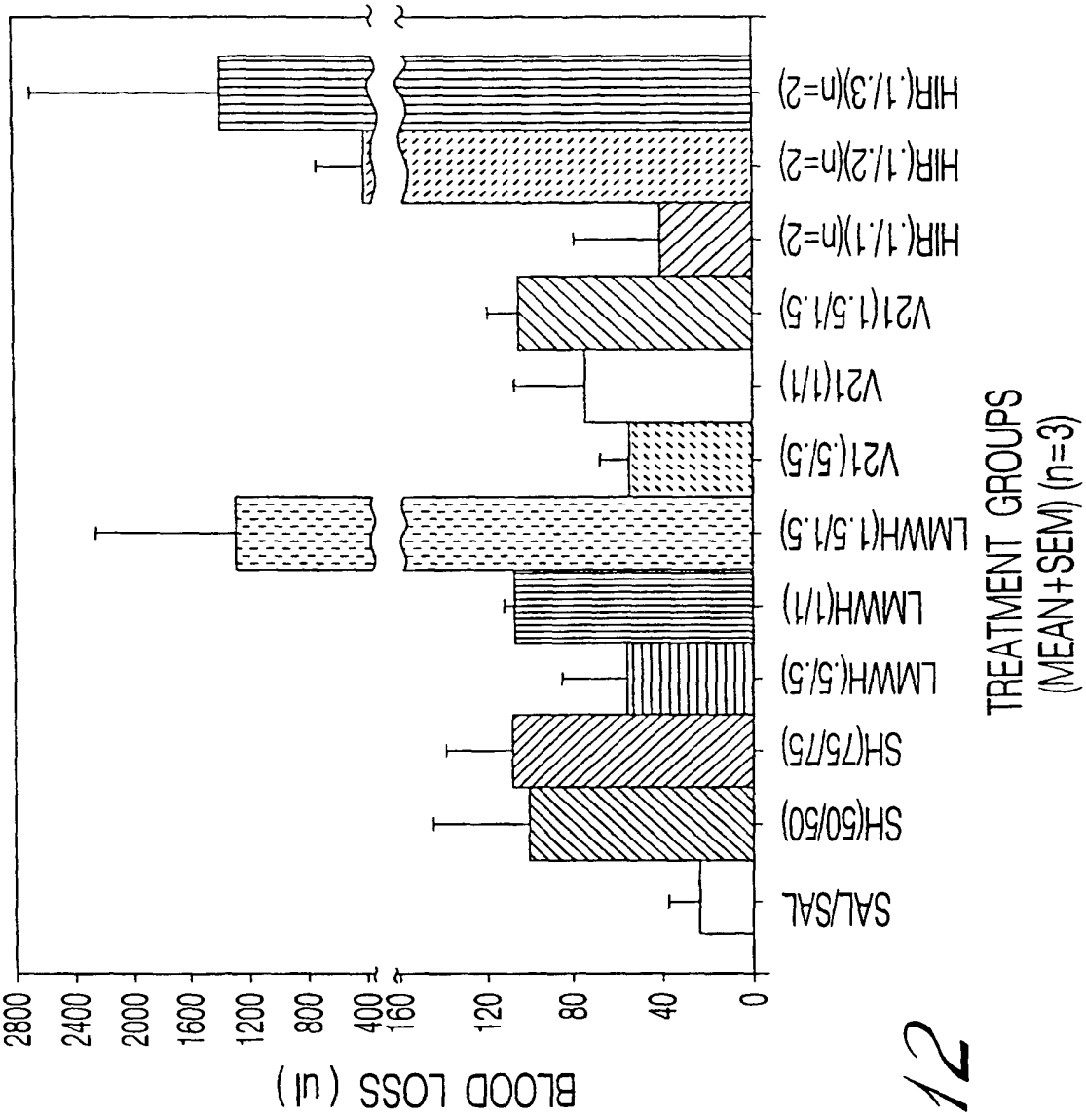
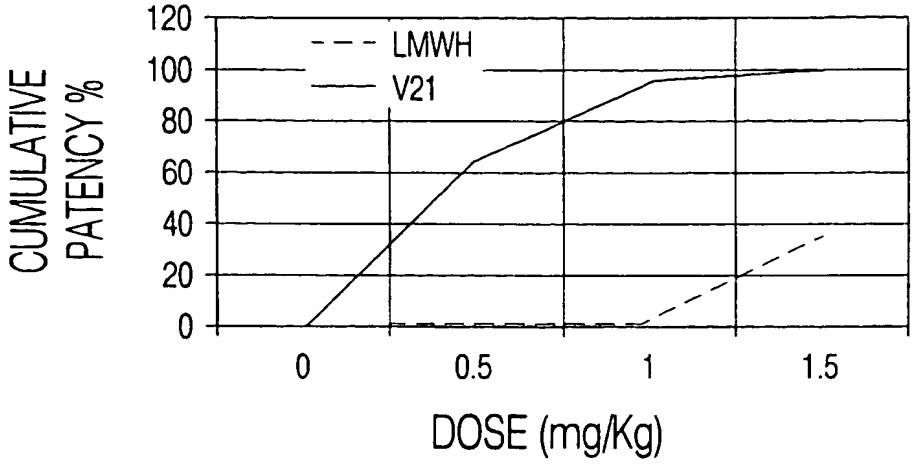


Fig. 12



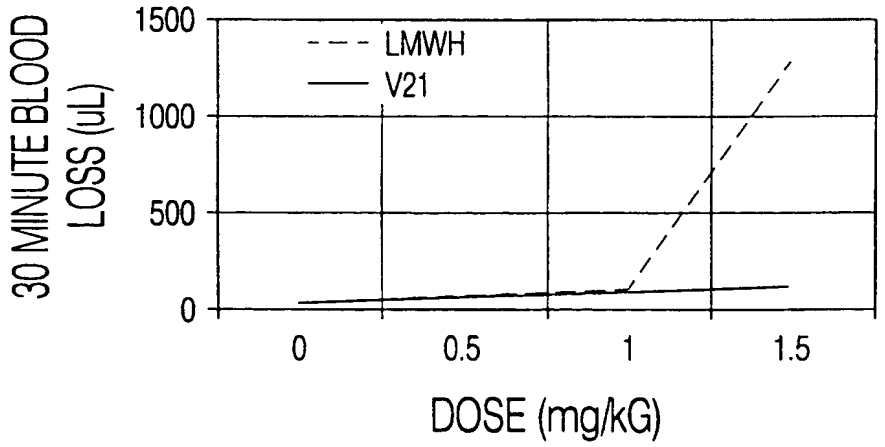


Fig. 13

	LMWH	V21		
0	21	21	0	0
0.5	24.7	23.1	12.4	1.12
1	24.3	30.7	2.37	10.1
1.5	39.4	52.1	2.98	2.31

Comparative effects of V21 and LMWH on APTT

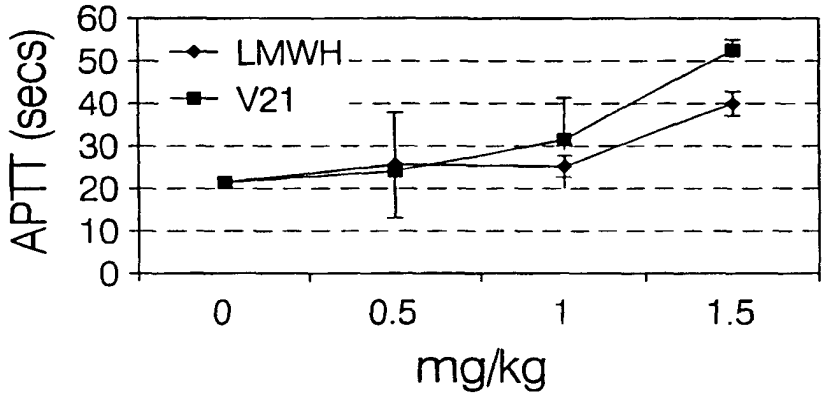


Fig. 14

LMWH	V21	lmwhse	V21 se	
0	0	0	0	0
0.5	1.1	0.8	0.19	0.32
1	2.8	1.5	0.91	0.32
1.5	2.6	2.8	0.06	0.36

Comparative effects of LMWH and V21 on the anti-Xa level

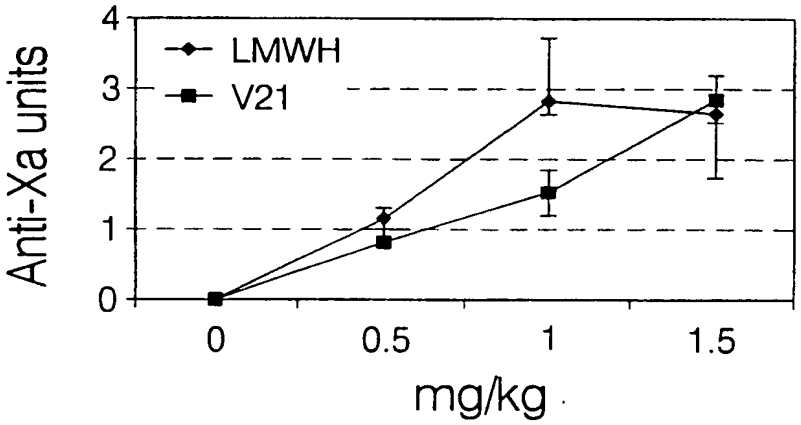


Fig. 15

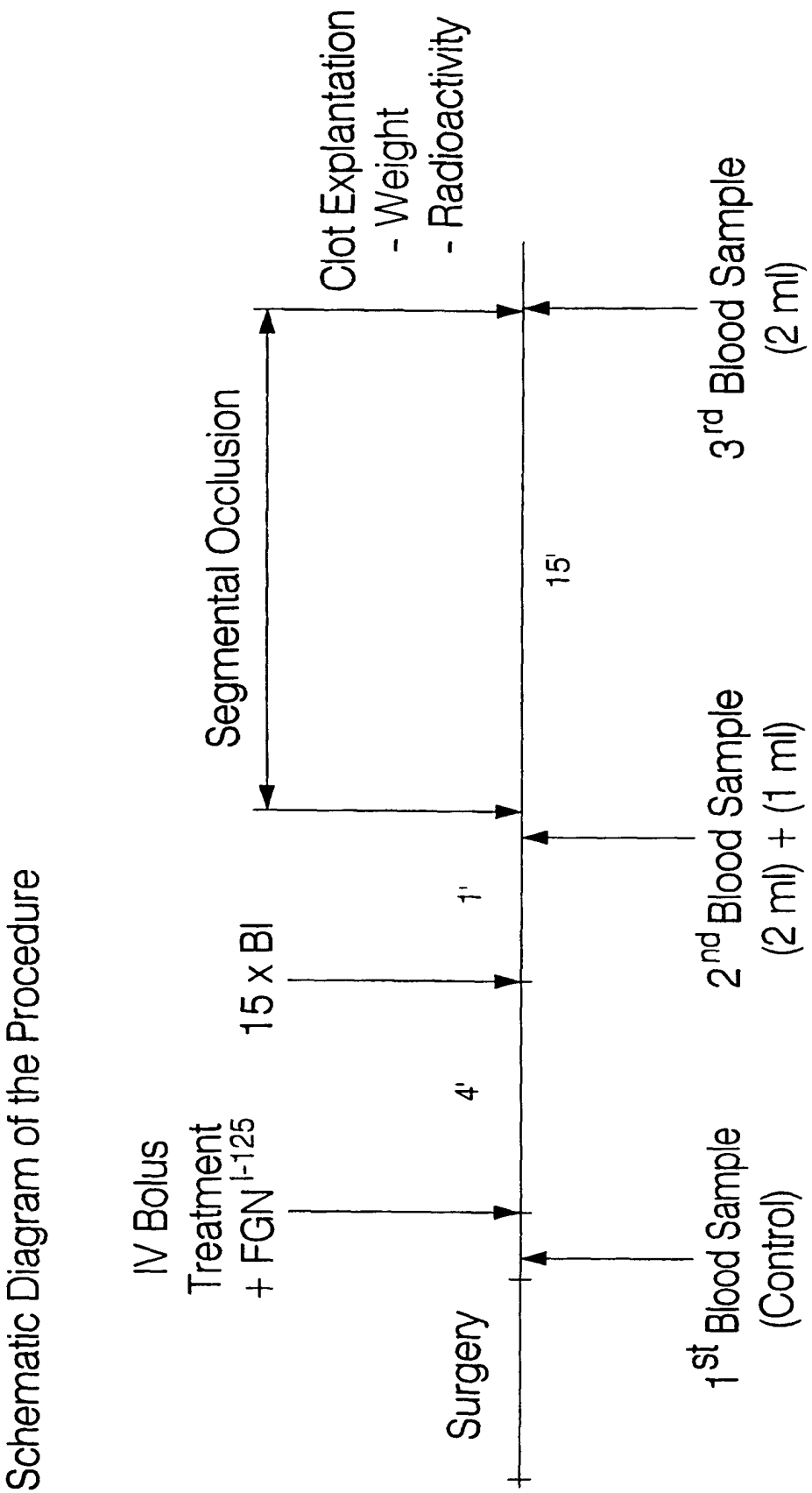


Fig. 16

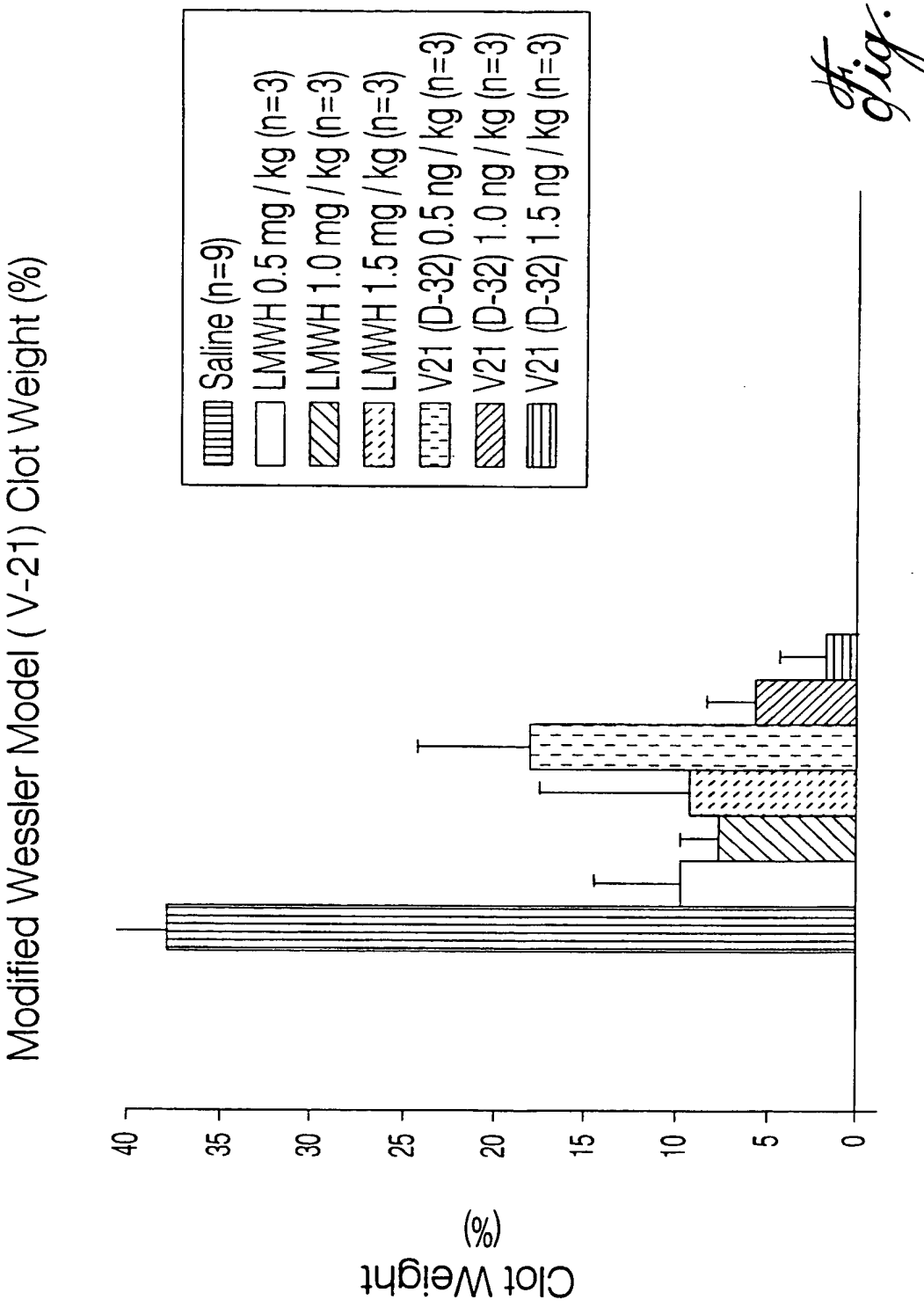


Fig. 17

	LMWH	V21		
0	37.8	37.8	3.7	3.7
0.5	9.6	18	4.7	6.1
1	7.5	5.6	2.1	2.6
1.5	9.1	1.7	8.2	0.6

Comparison of LMWH and V21:Prophylaxis model

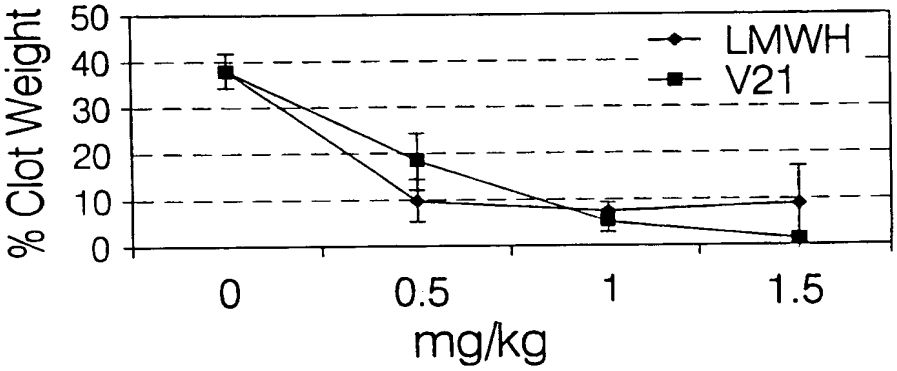


Fig. 18

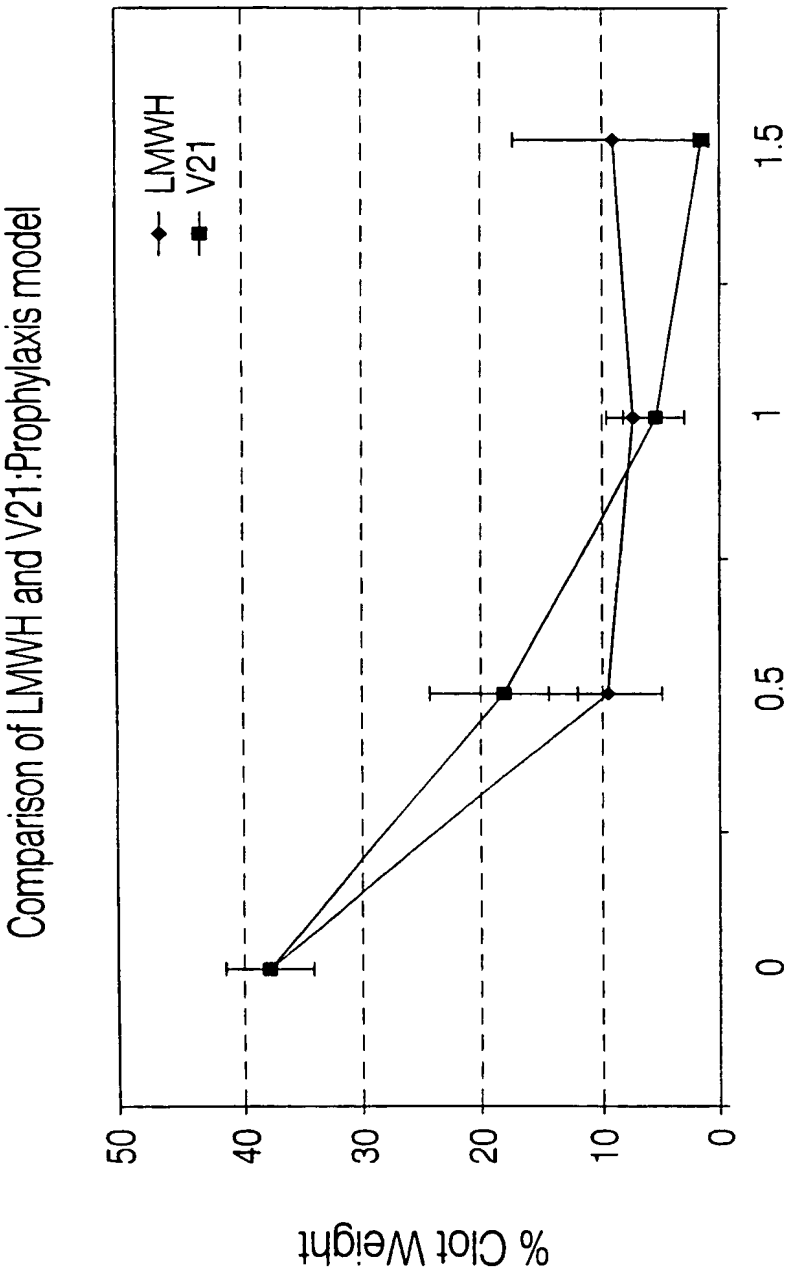


Fig. 19

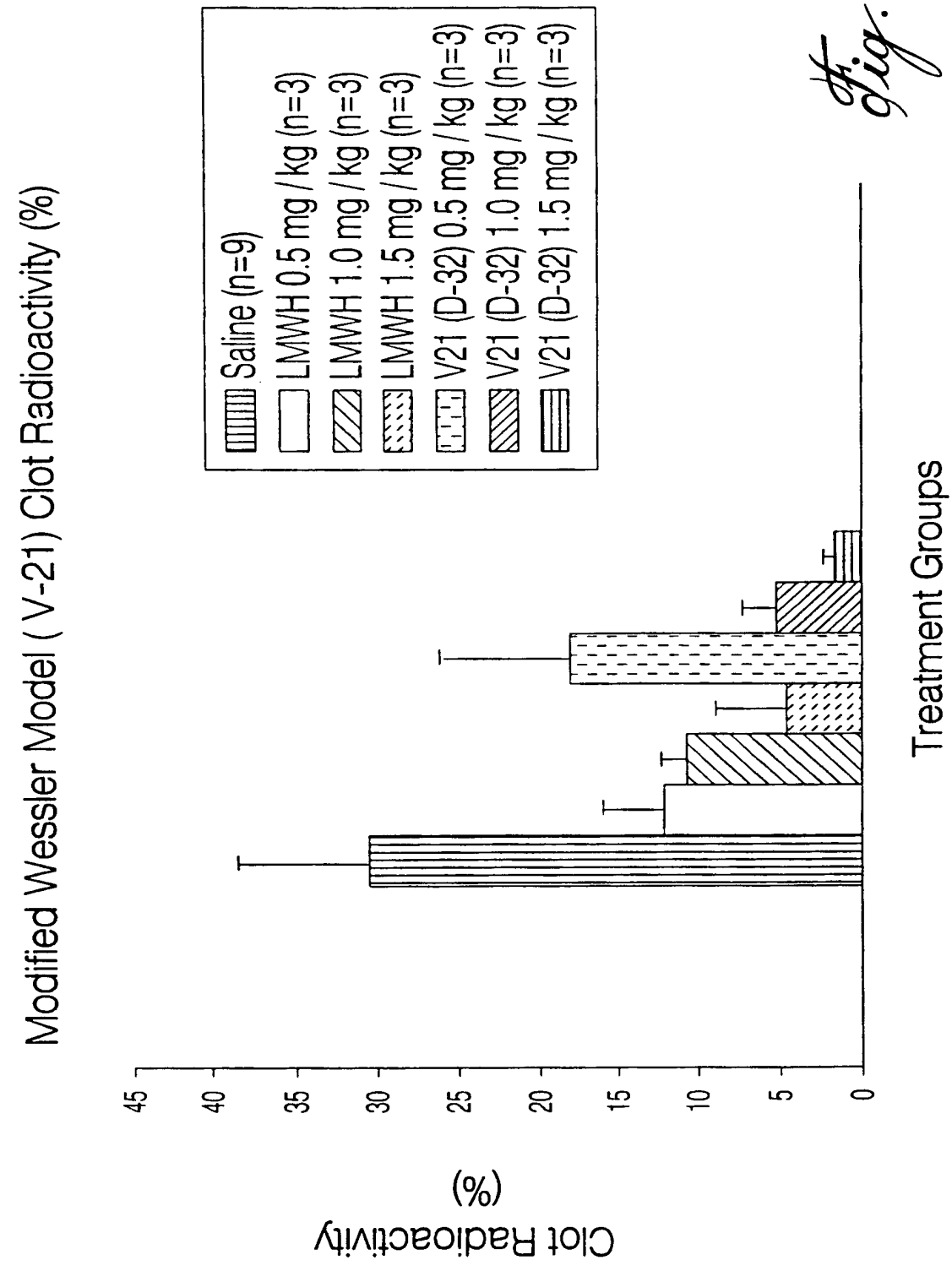


Fig. 20

	LMWH	V21	se	se
0	30	30	8	8
0.5	12	18	3.8	7.8
1	11	5	1.7	2.1
1.5	4.5	1.6	4.2	0.7

Comparison of LMWH and V21:Prophylaxis Model

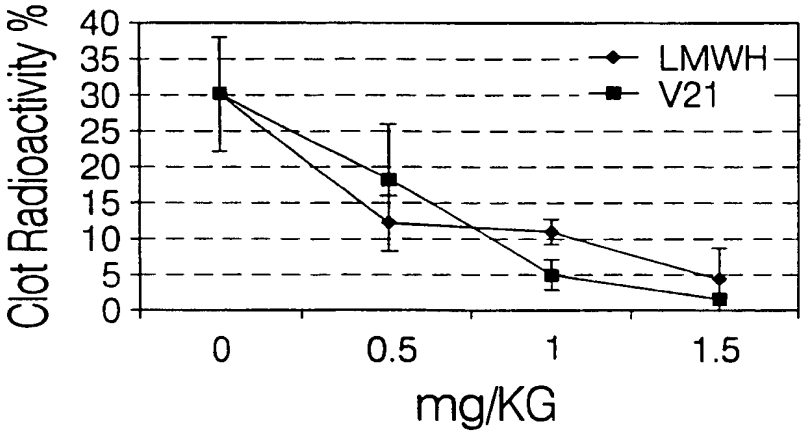


Fig. 21

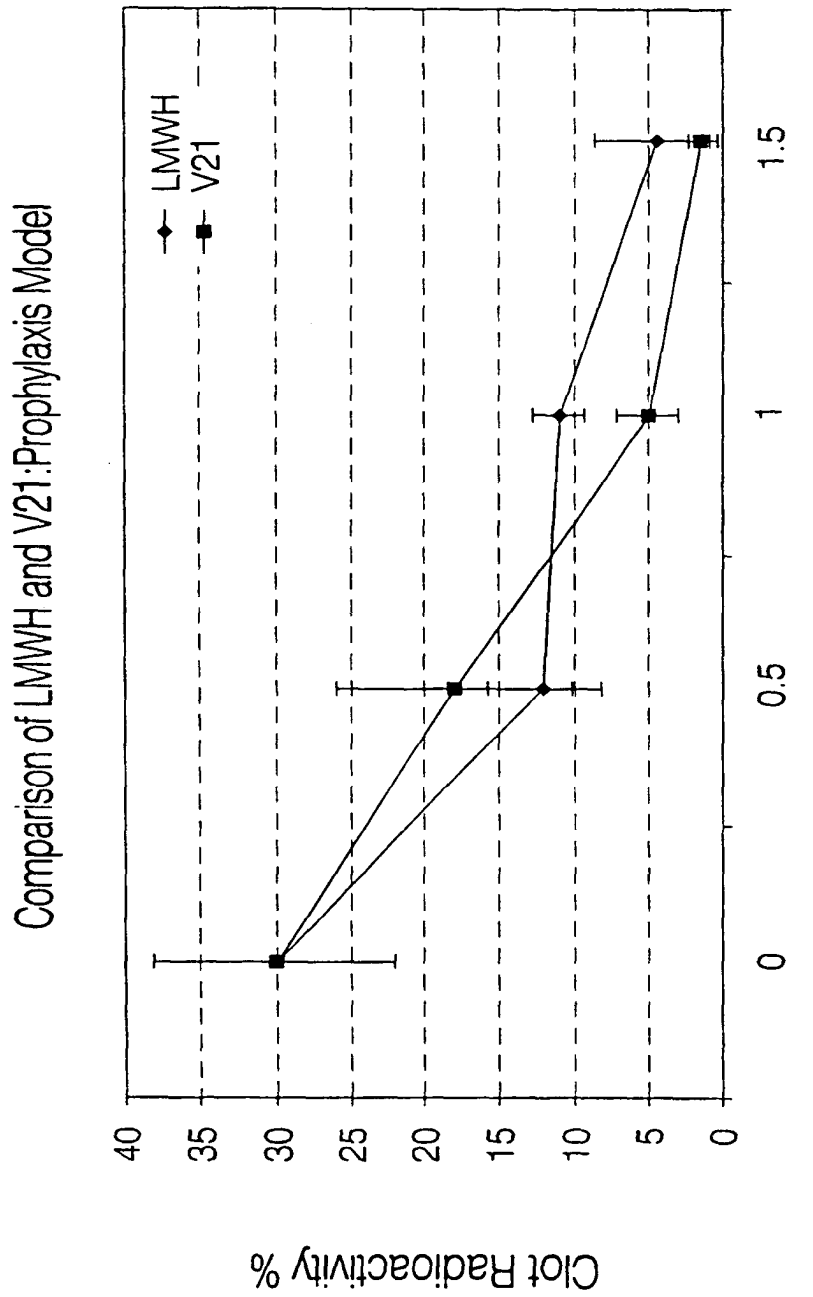


Fig. 22

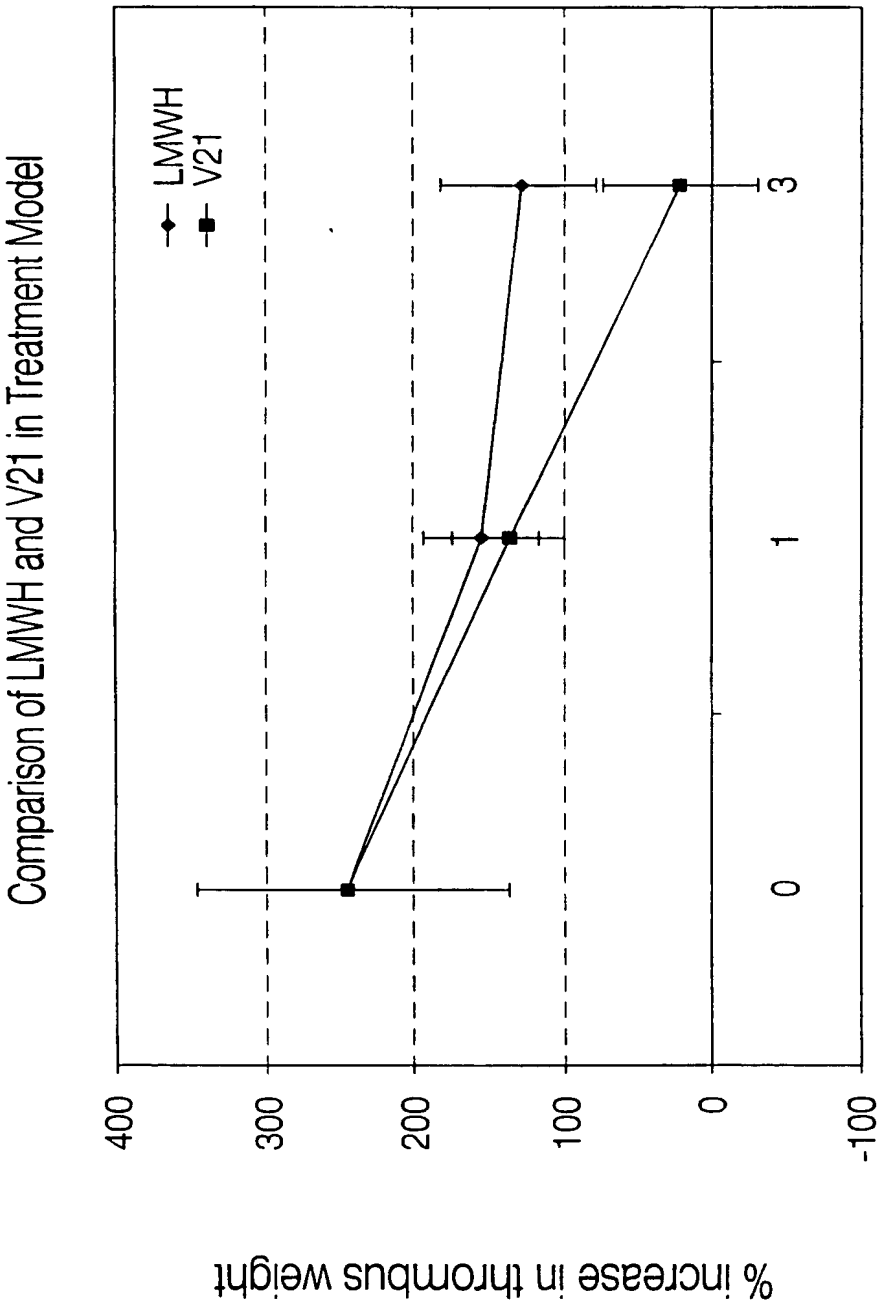


fig. 23

	LMWH	V21		
0	242	242	104	104
1	155	137	37	30
3	129	21	52	31

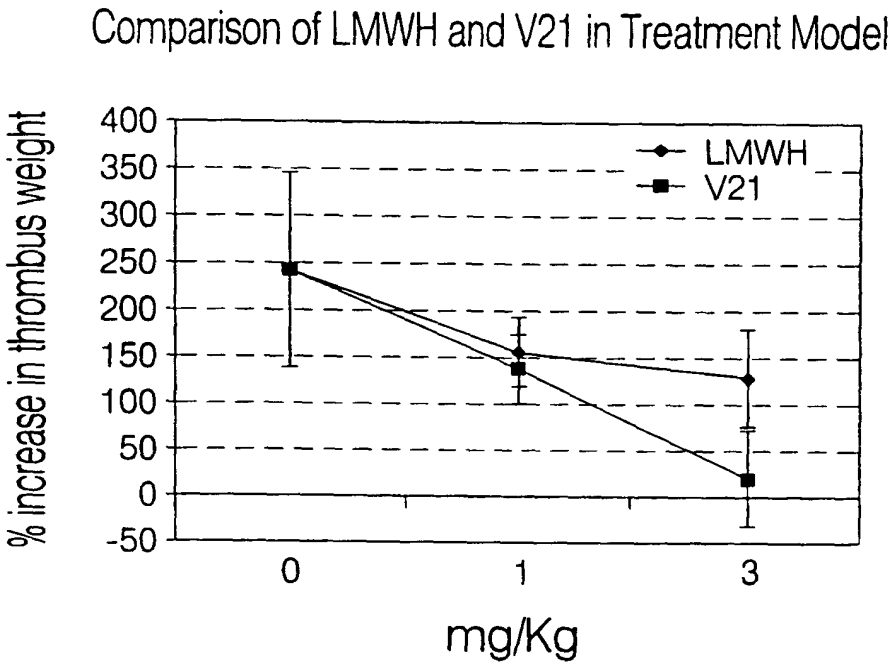


Fig. 24

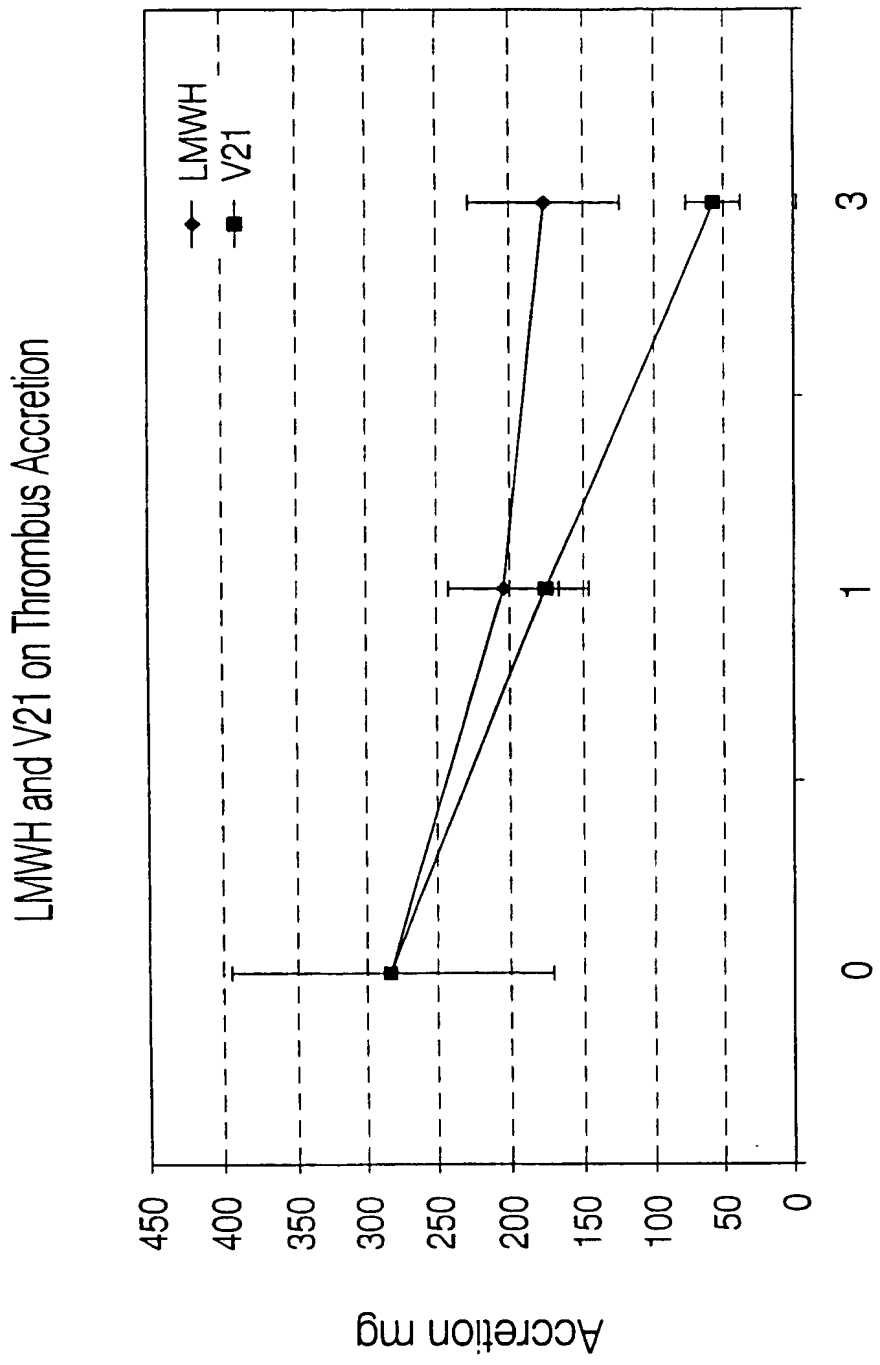


Fig. 25

	LMWH	V21		
0	282	282	111	111
1	202	174	37	29
3	174	57	51	20

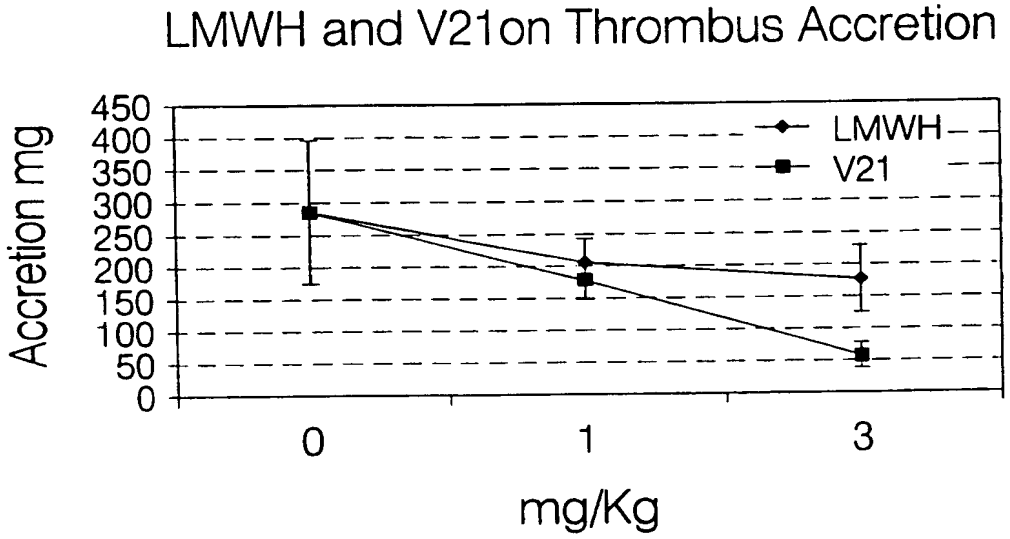


Fig. 26

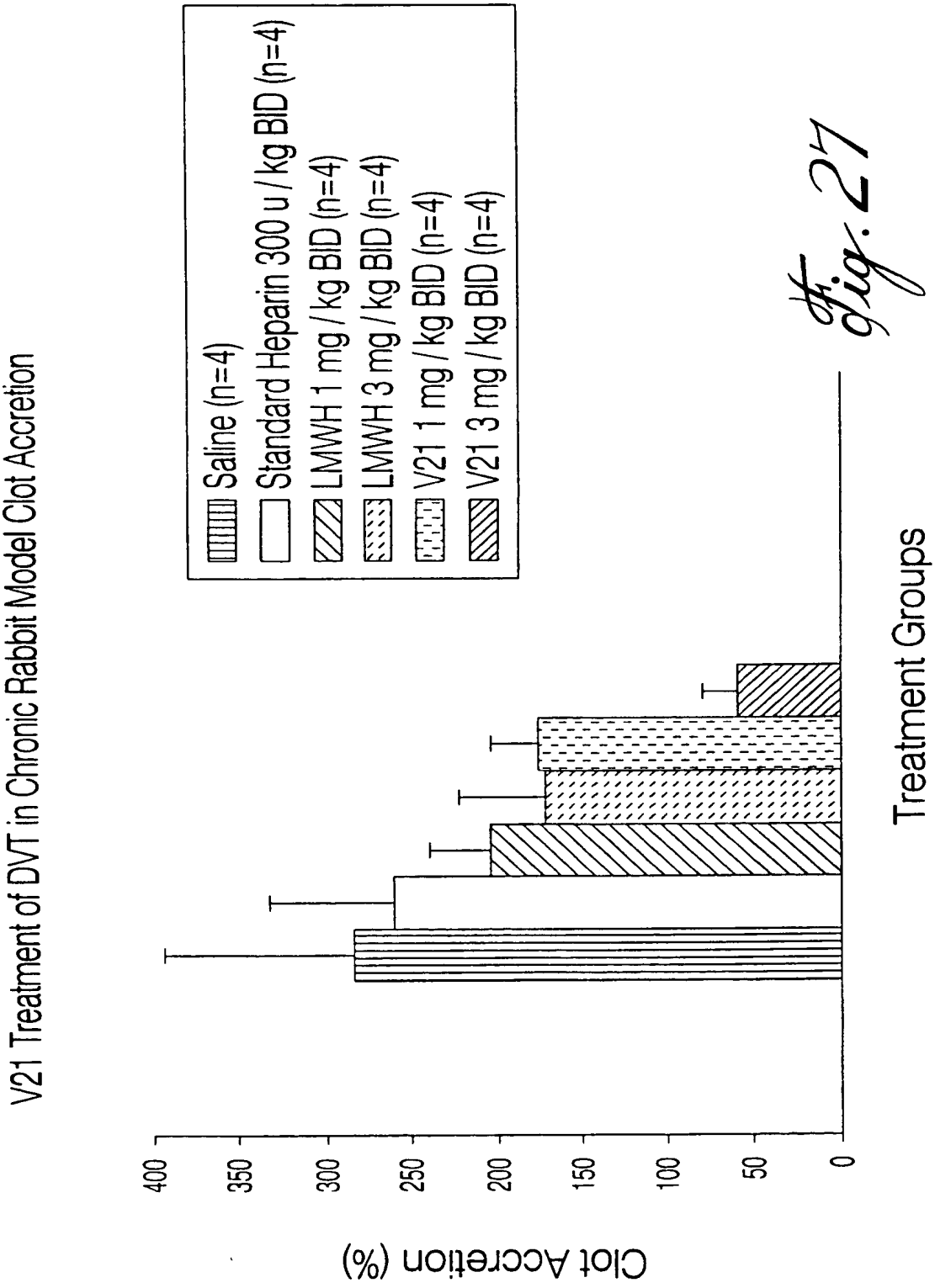


Fig. 27

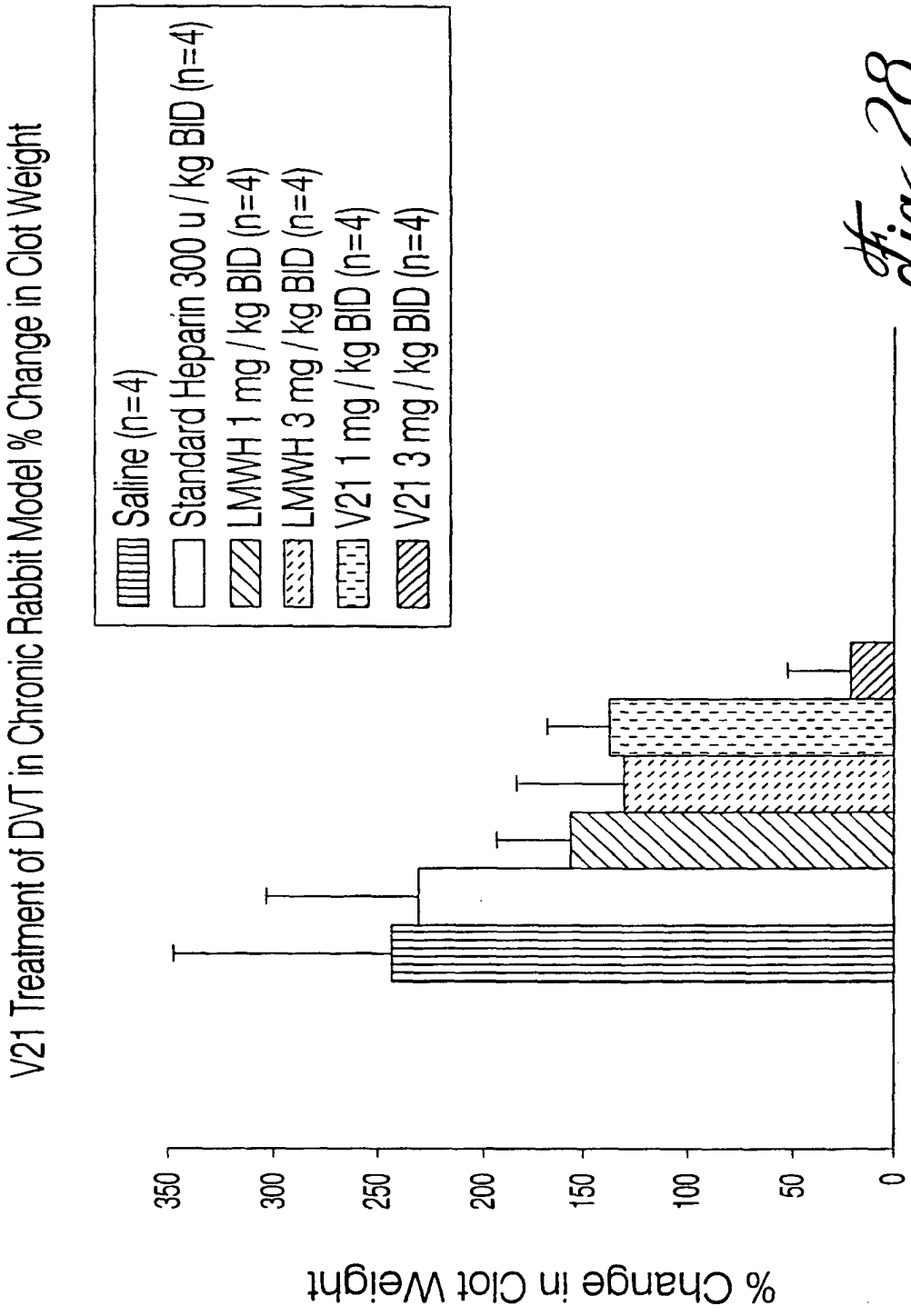


Fig. 28

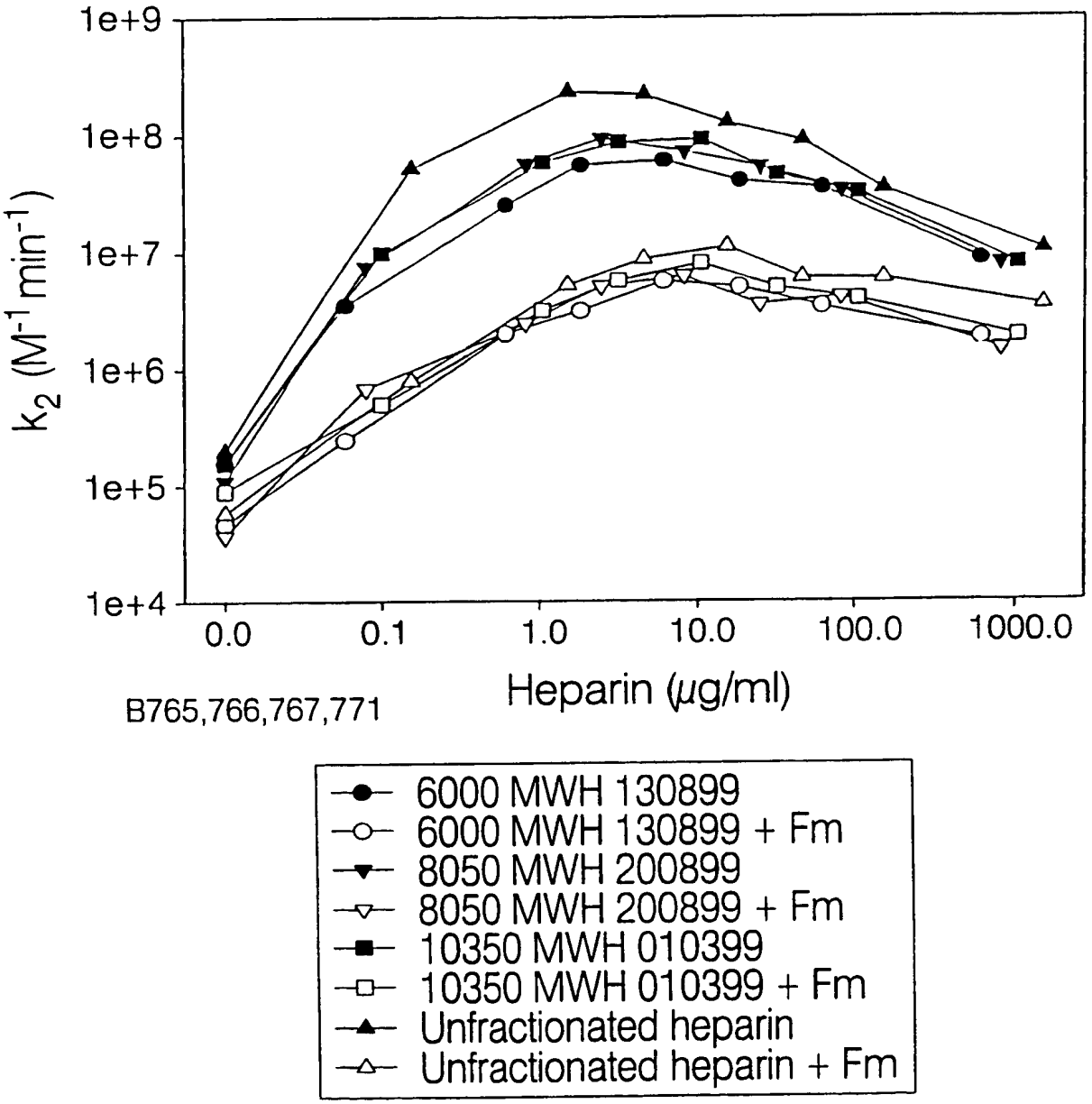


Fig. 29

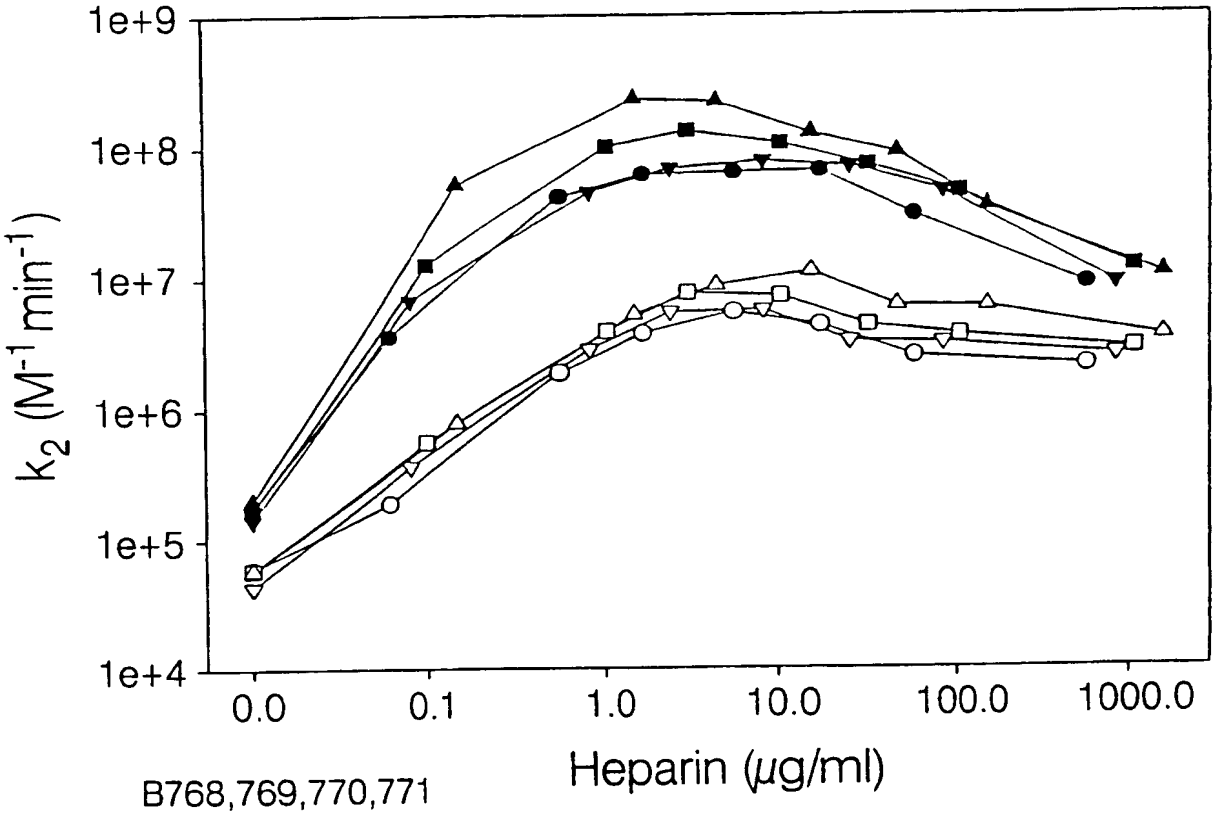


Fig. 30

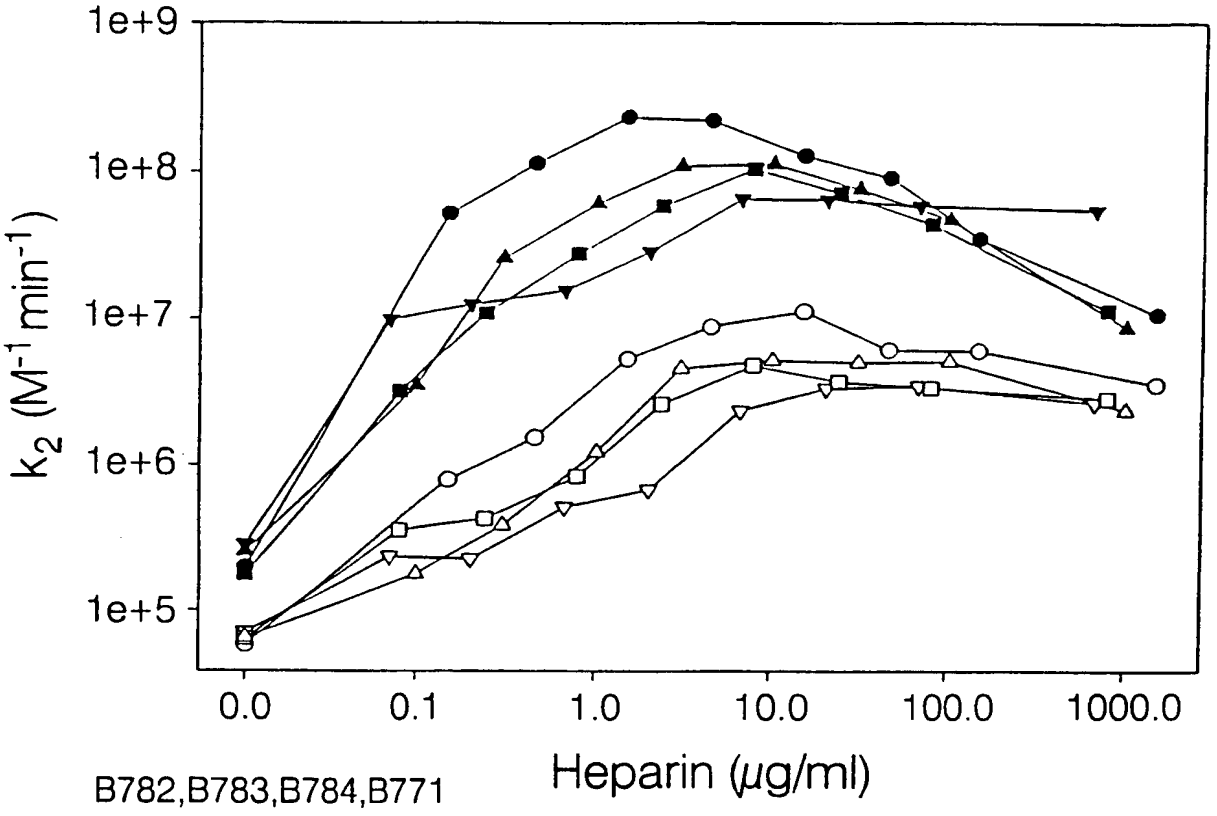


Fig. 31

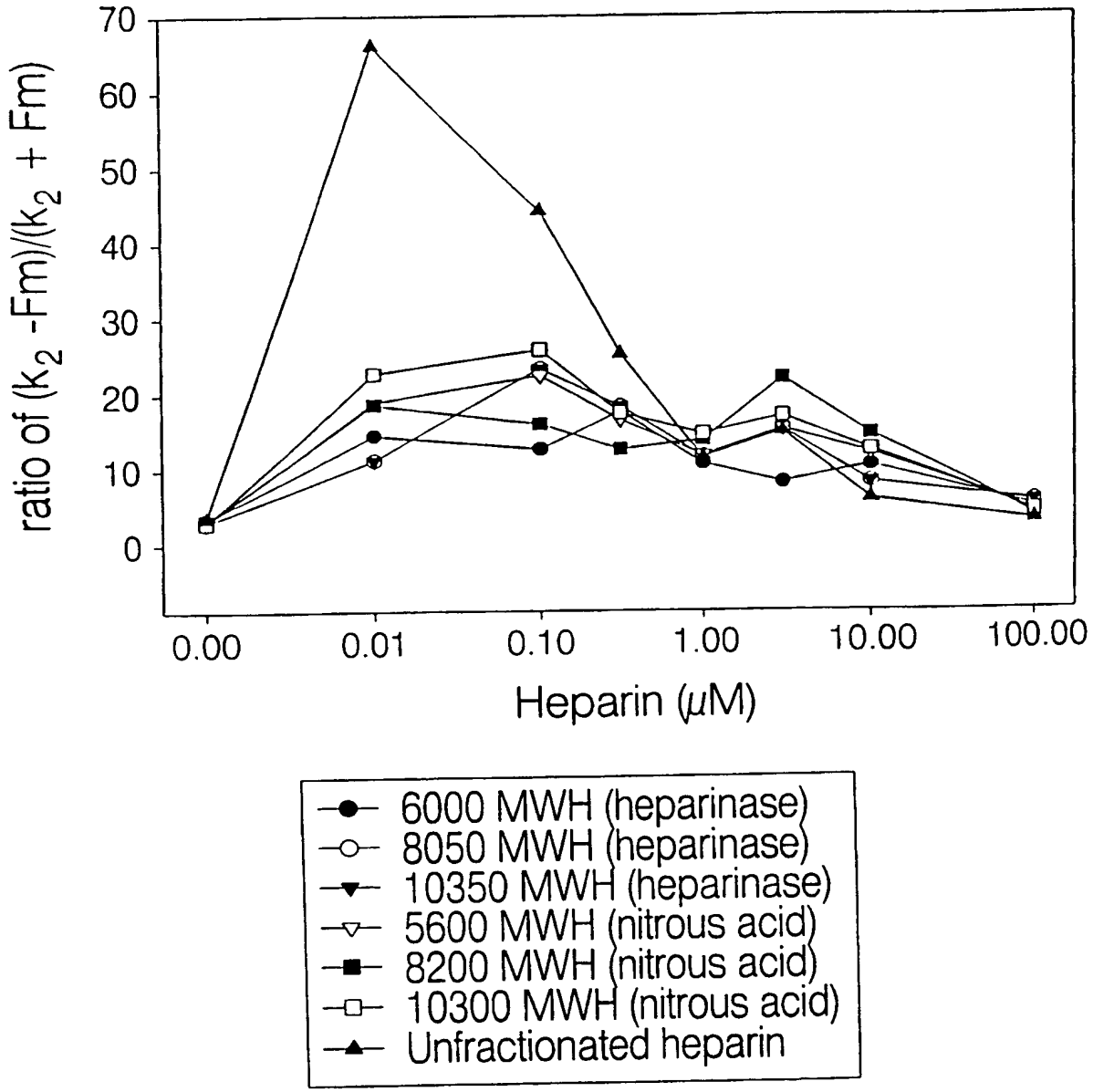


Fig. 32

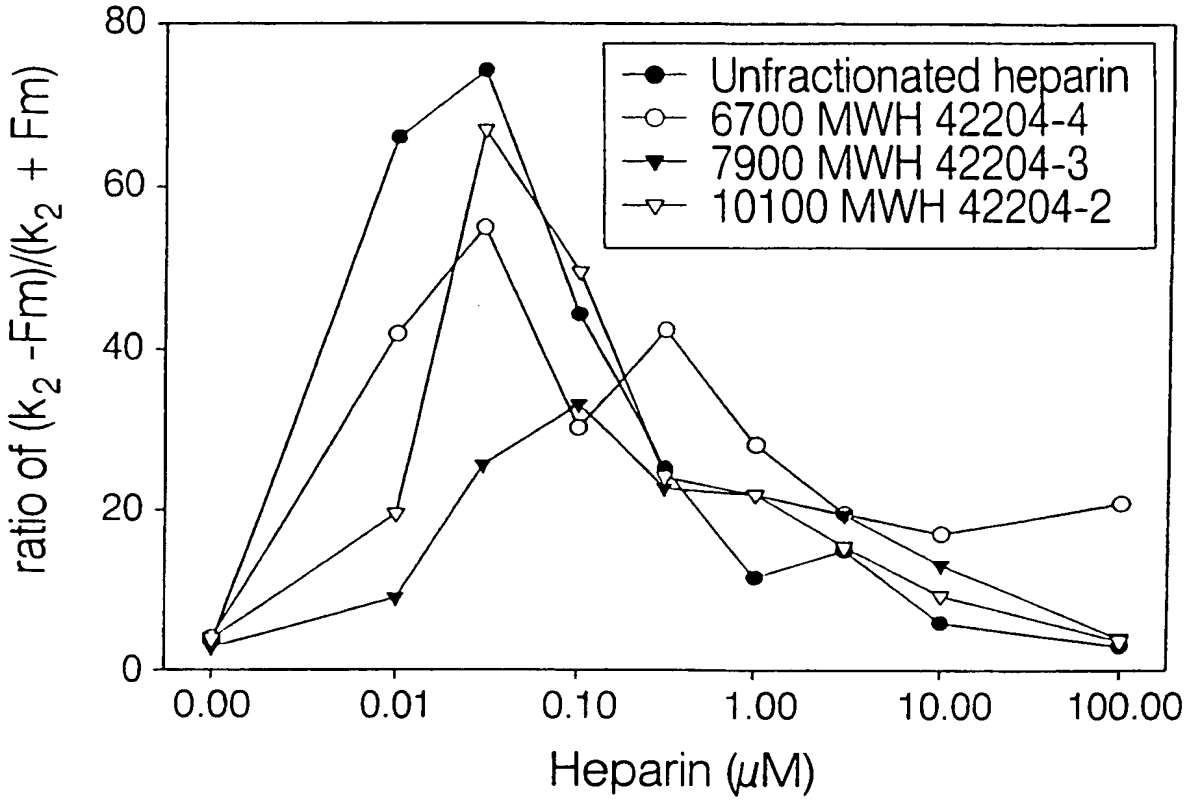


Fig. 33

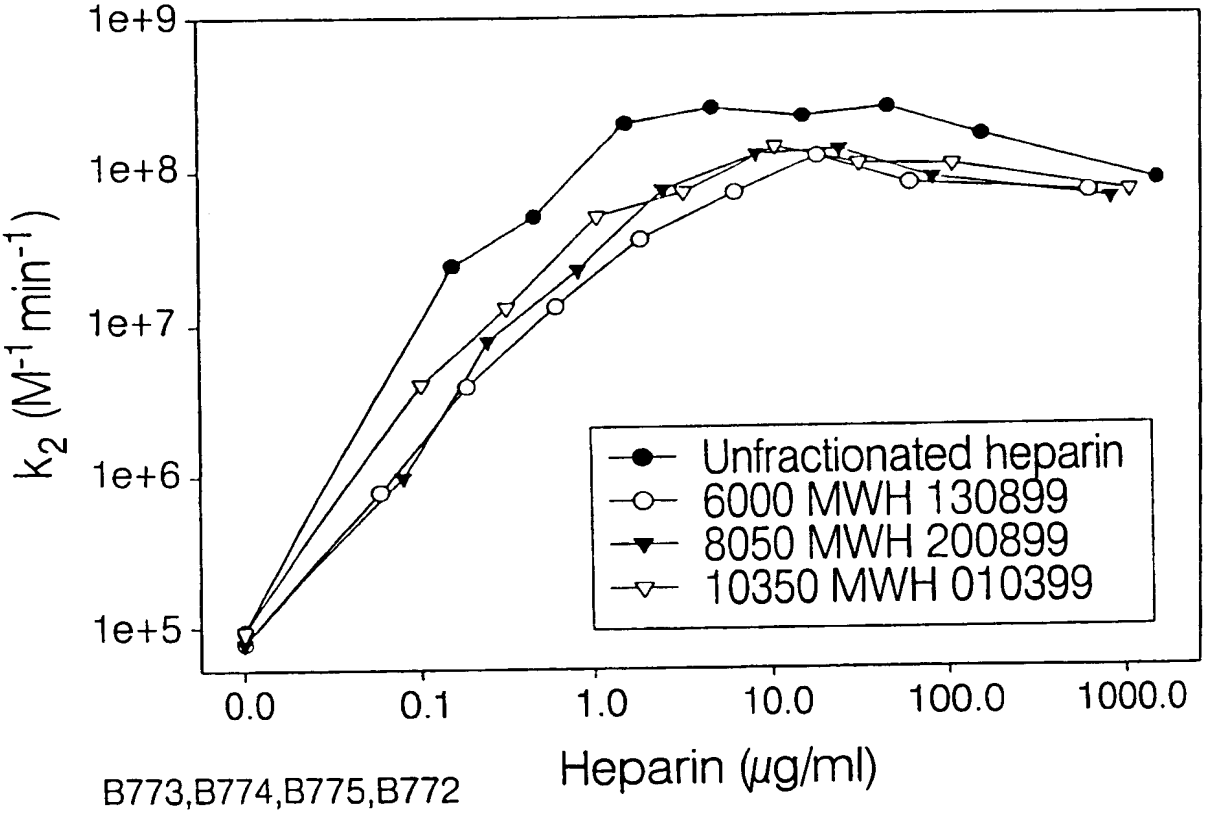


Fig. 34

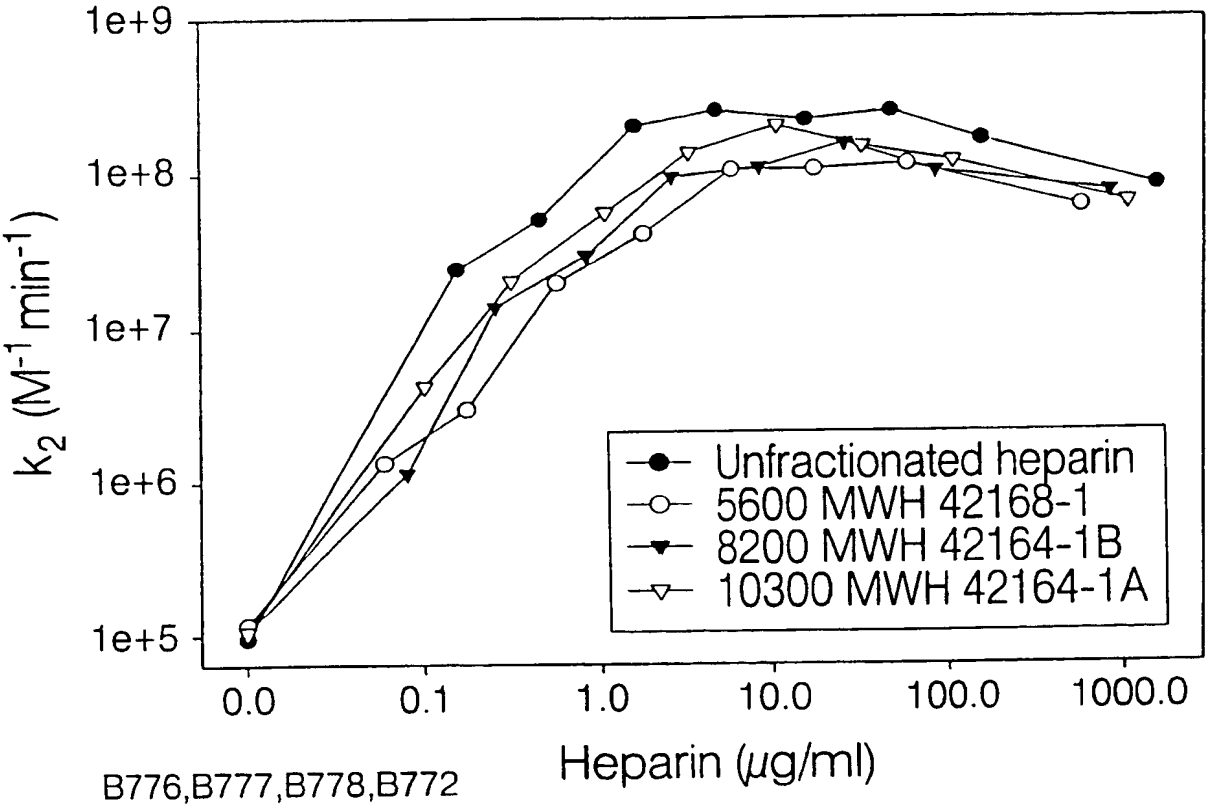


Fig. 35

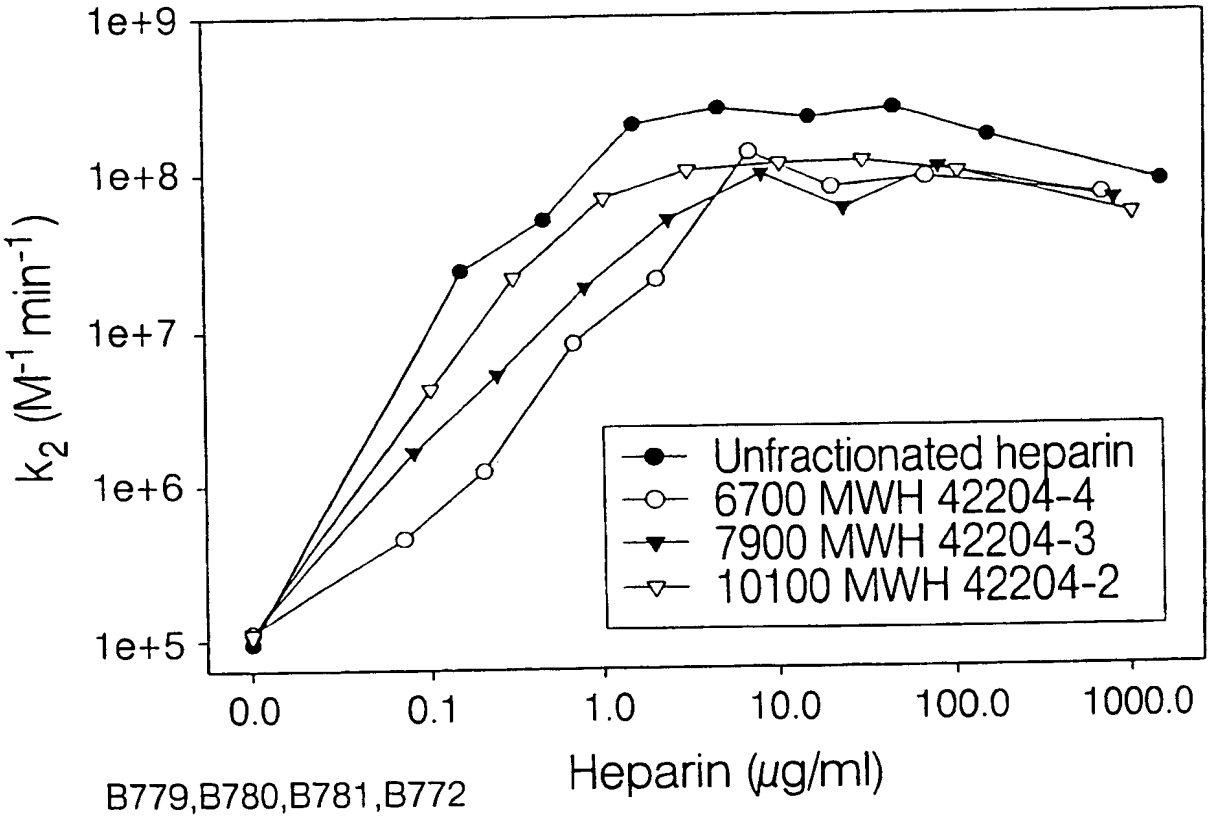


Fig. 36

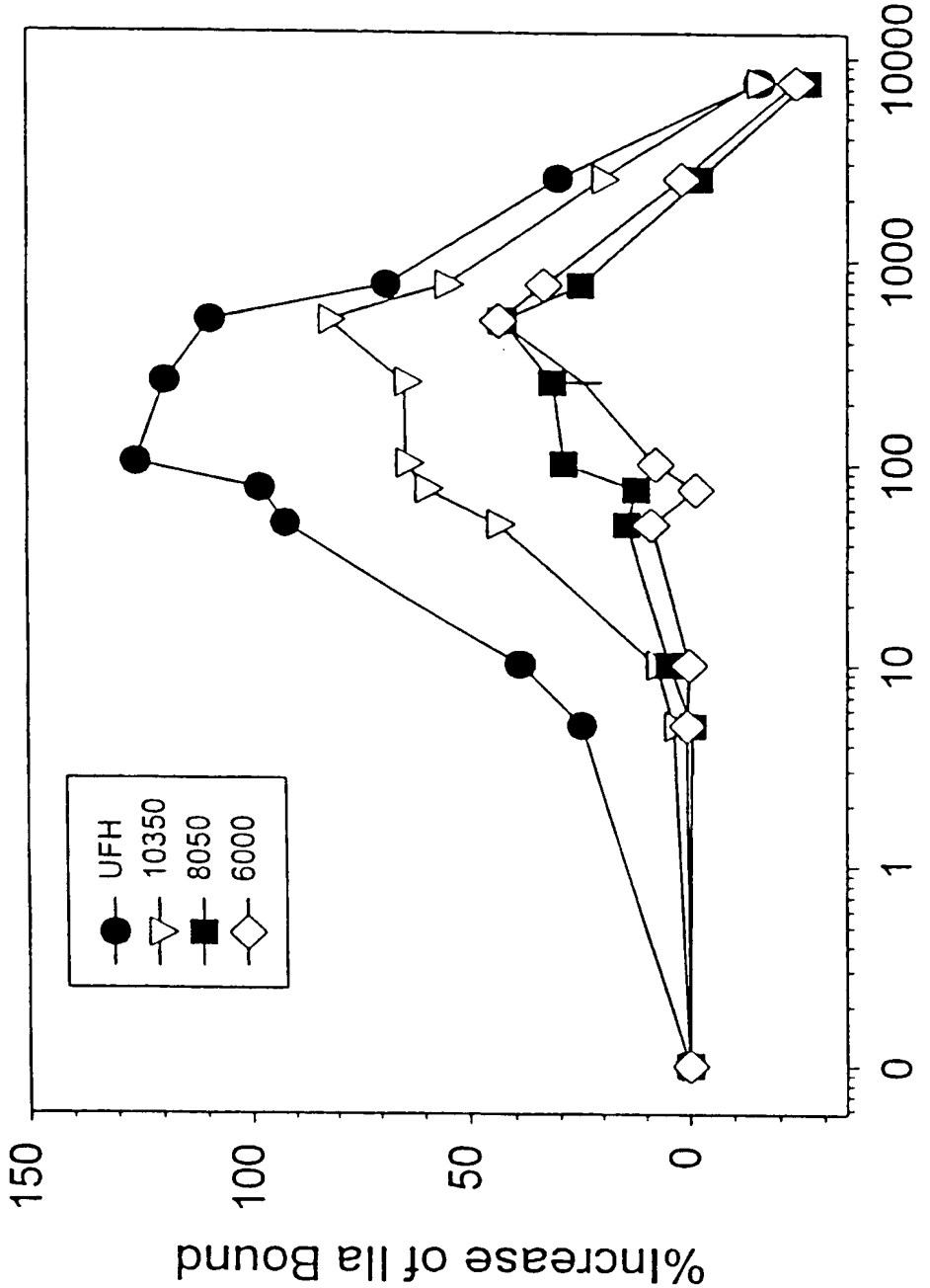


fig. 37

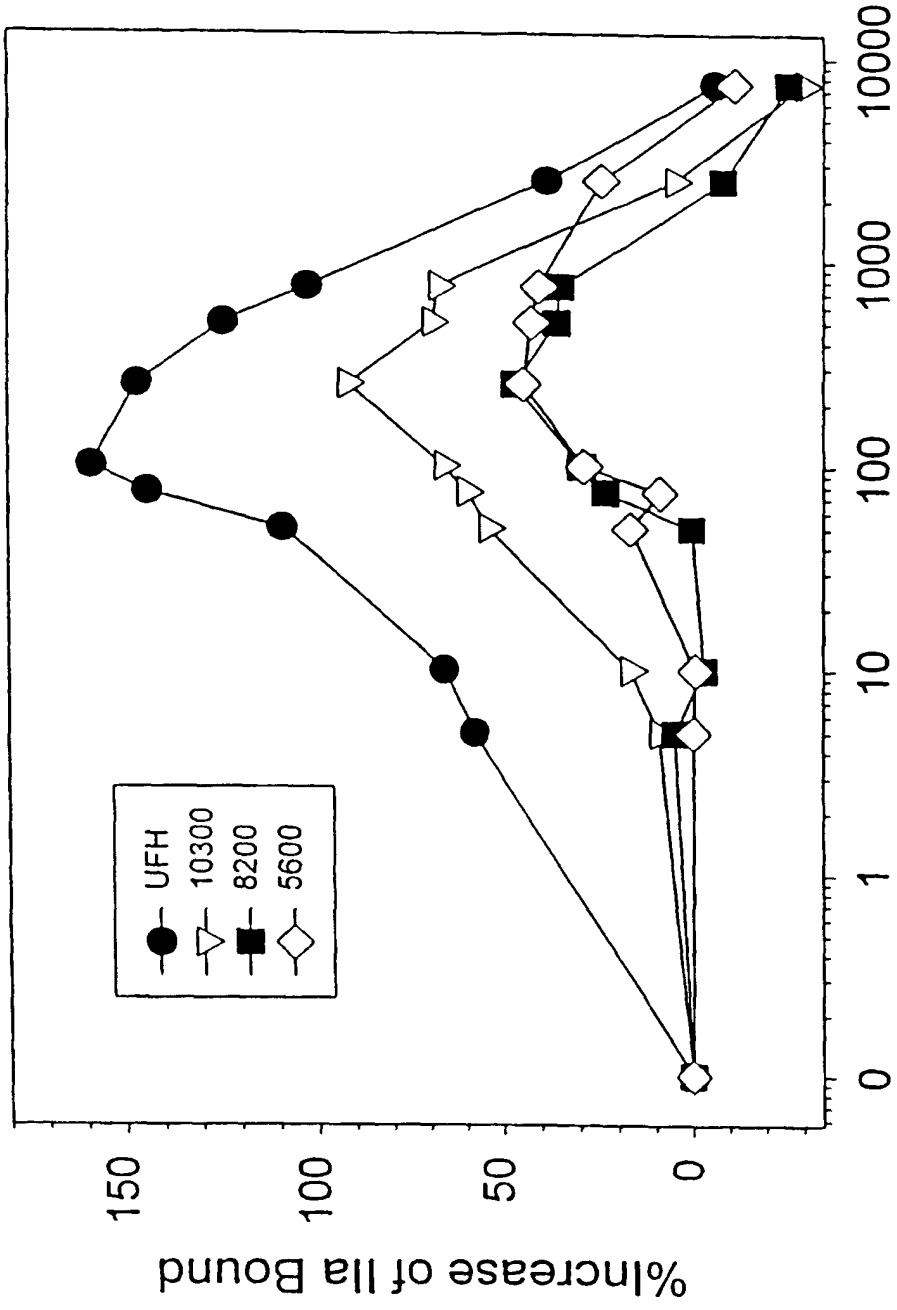


fig. 38

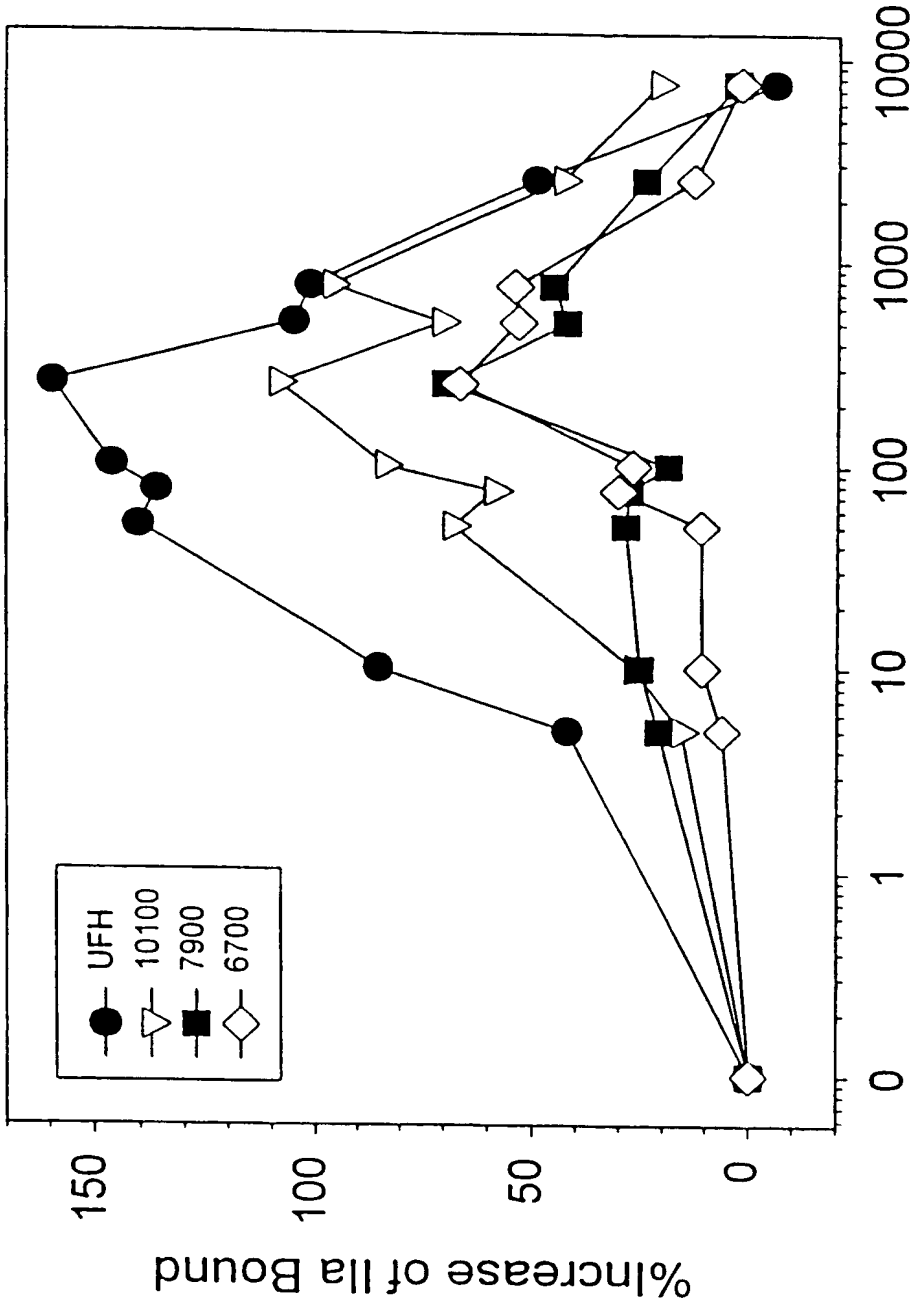


Fig. 39

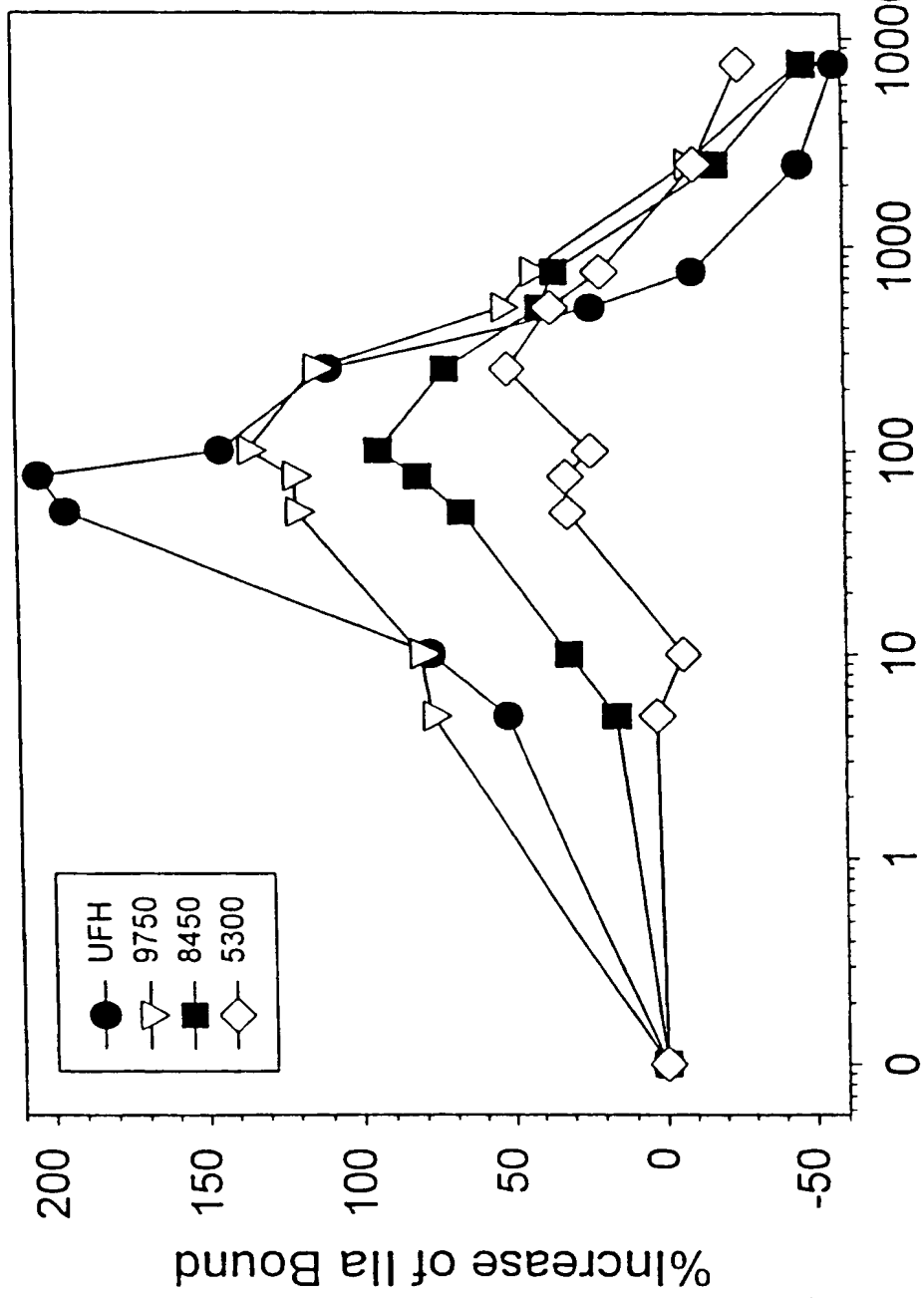


Fig. 40

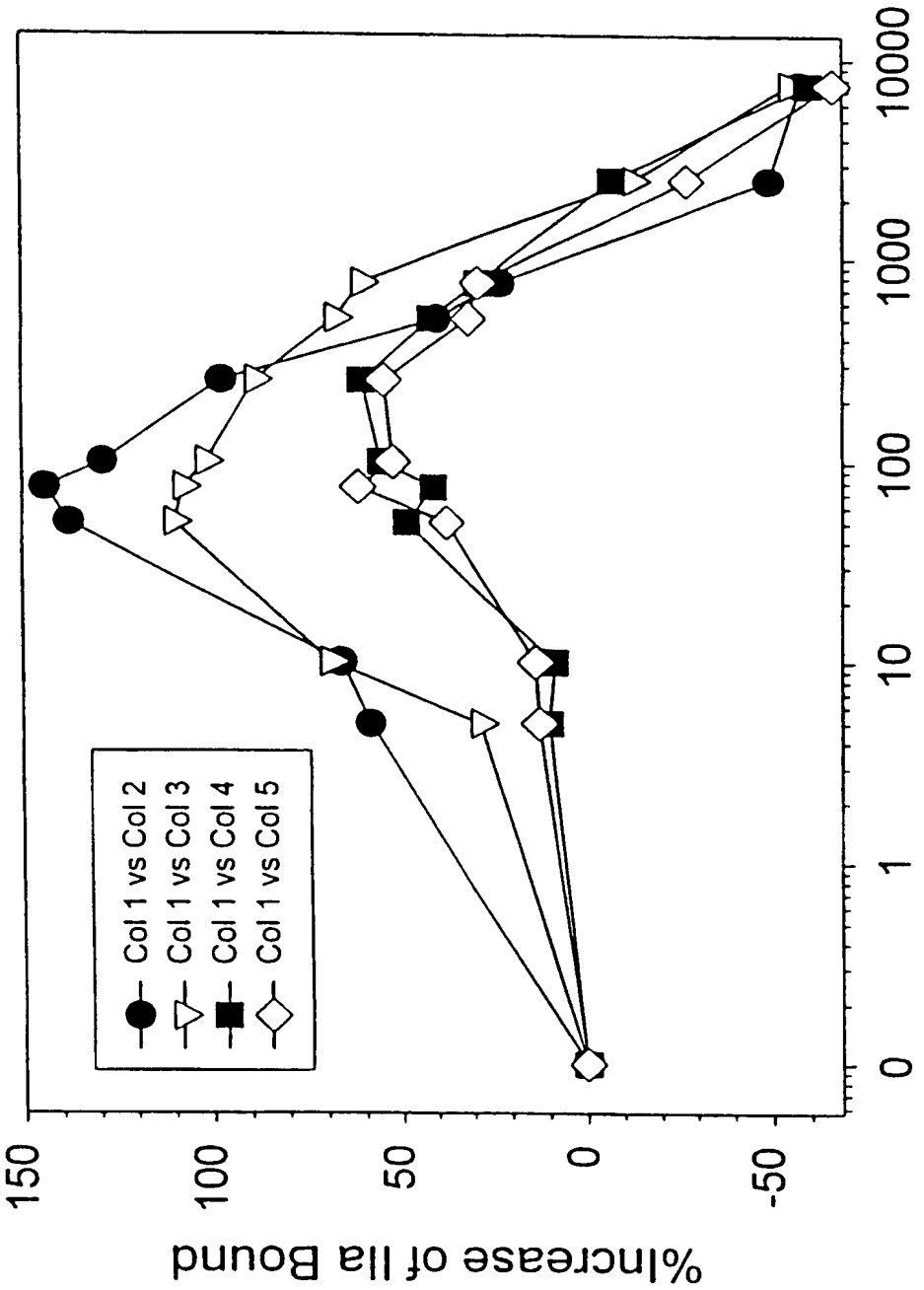


Fig. 41

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/CA 00/00774

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08B37/10 A61K31/727

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C08B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 101 141 A (HEPAR INDUSTRIES INC.) 22 February 1984 (1984-02-22) page 5, line 9 - line 13 claims	1,10
A	WO 98 55515 A (HAMILTON CIVIC HOSPITALS RESEARCH DEVELOPMENT INC.) 10 December 1998 (1998-12-10) claims	1-34
A	EP 0 244 235 A (NOVO INDUSTRIA/S) 4 November 1987 (1987-11-04) examples 1,2 tables I,II	1,10,16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 2000	08/11/2000

Name and mailing address of the ISA	Authorized officer
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3

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/CA 00/00774

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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(B) EFFECT OF HEPARIN ON THE AFFINITY OF α-THROMBIN FOR FIBRIN

HEPARIN (μM)	Kd (μM)
0	3.22
0.1	0.25
0.25	0.15
1	0.79
20	4.26

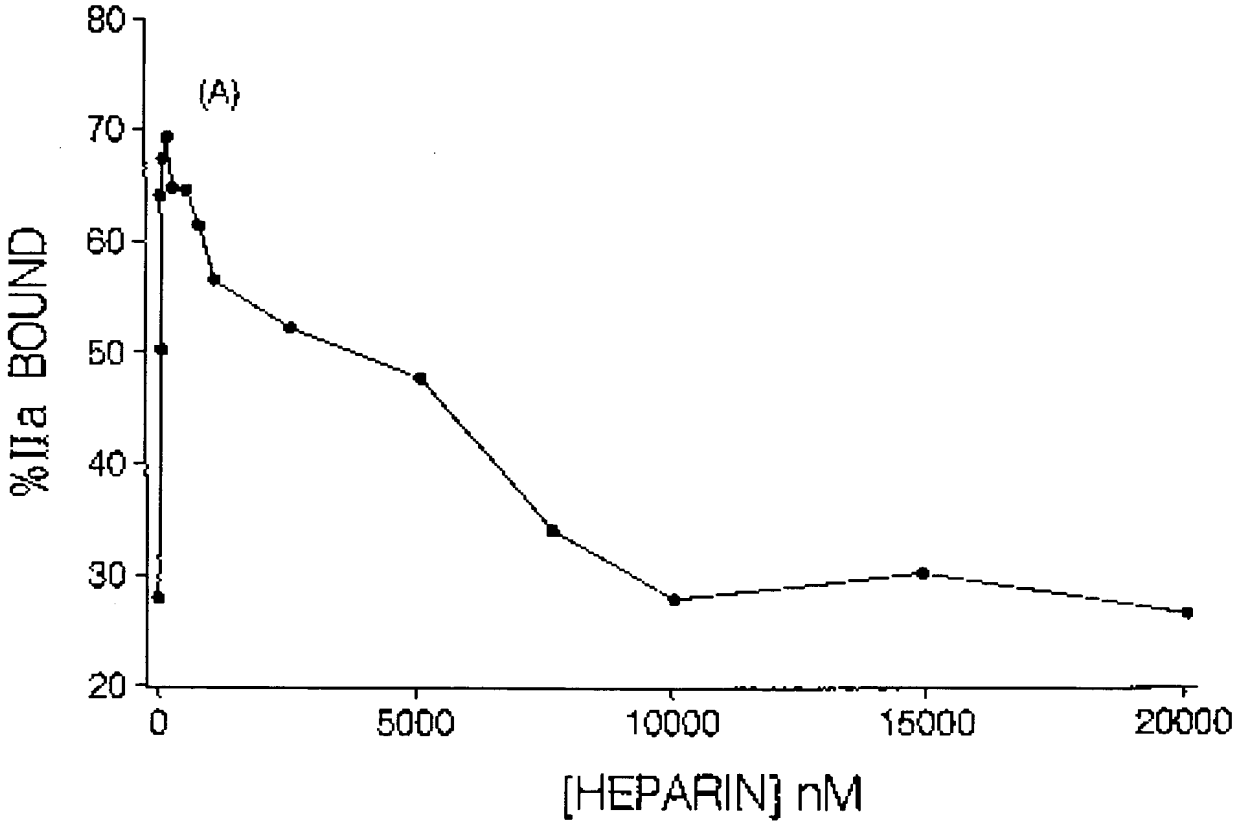

Fig. 1

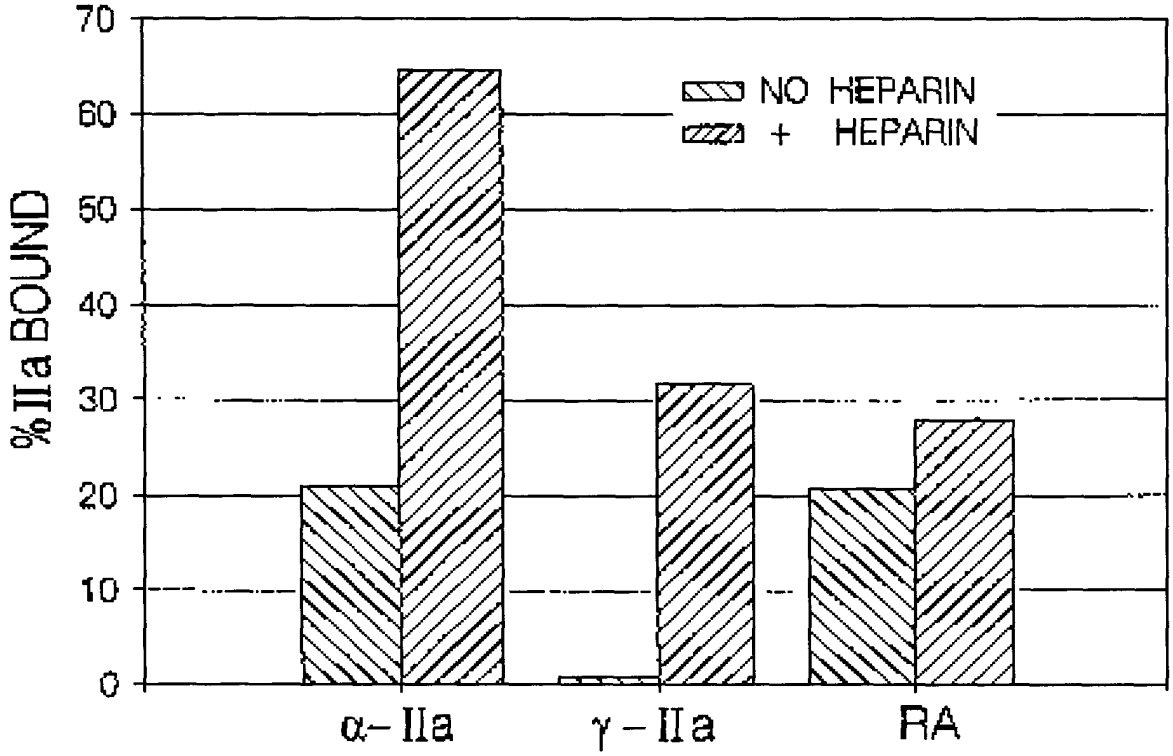

Fig. 2

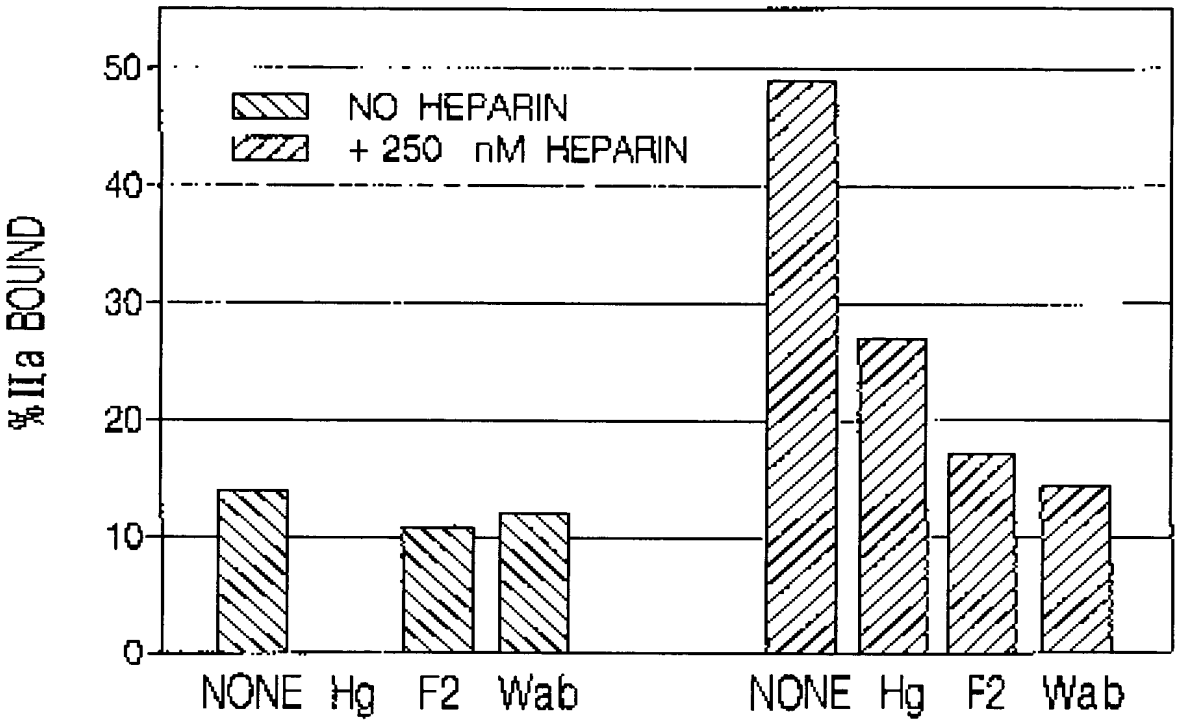

Fig. 3

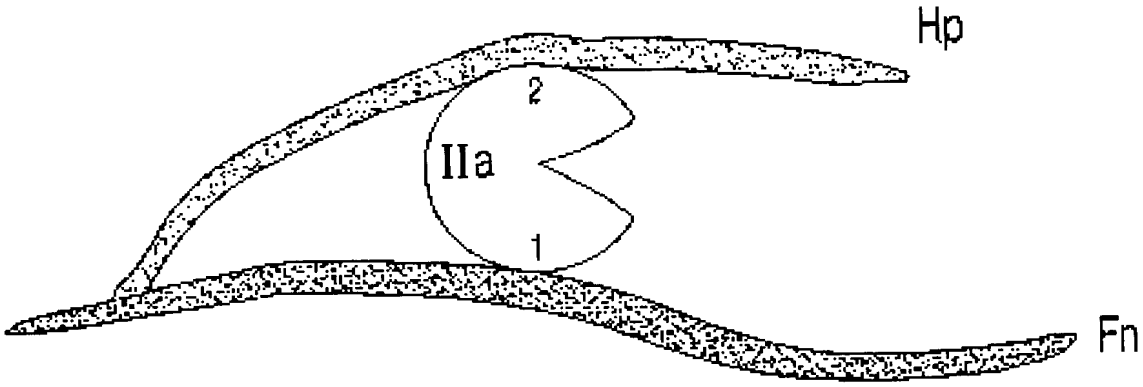

Fig. 4

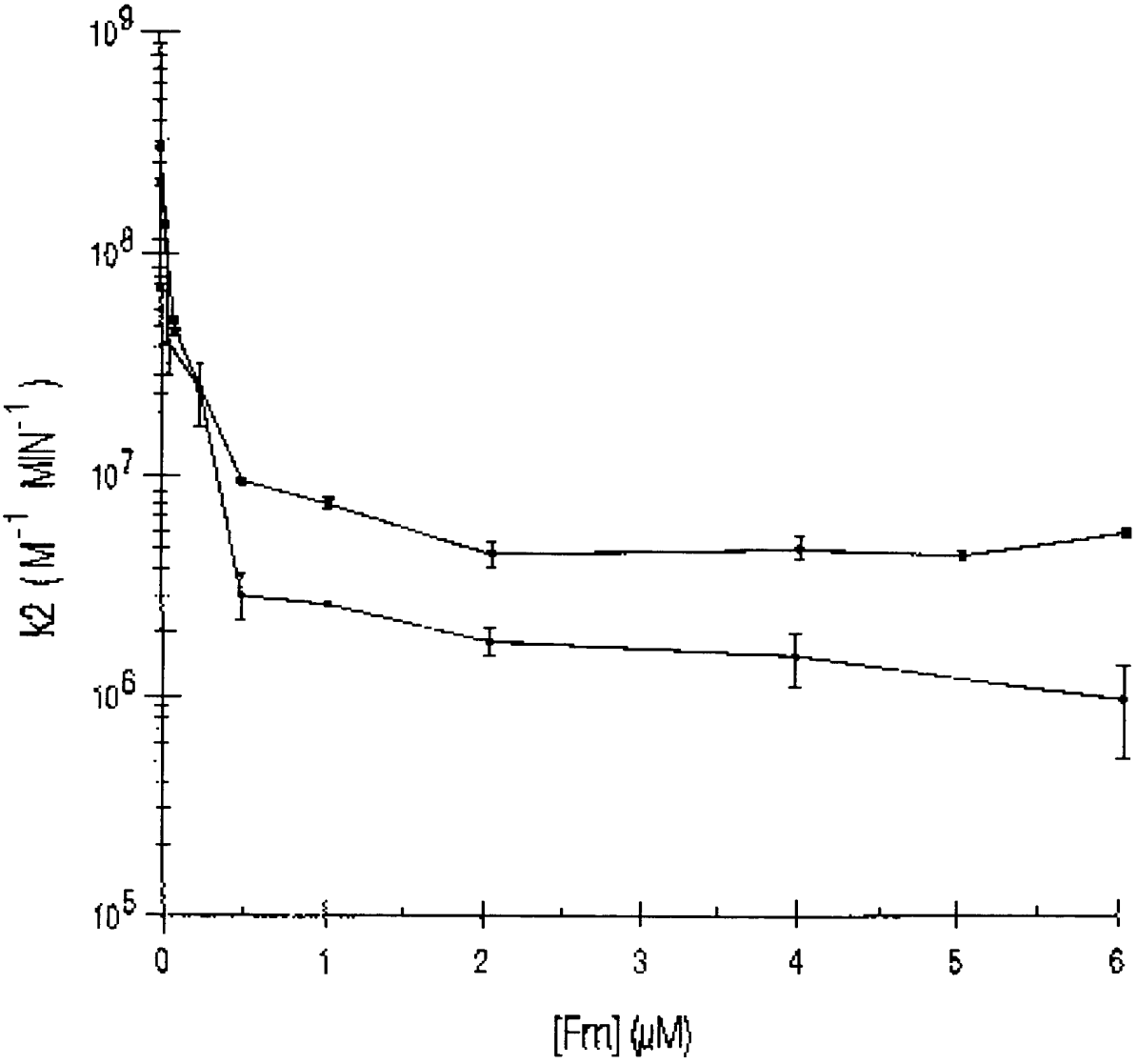

Fig. 5

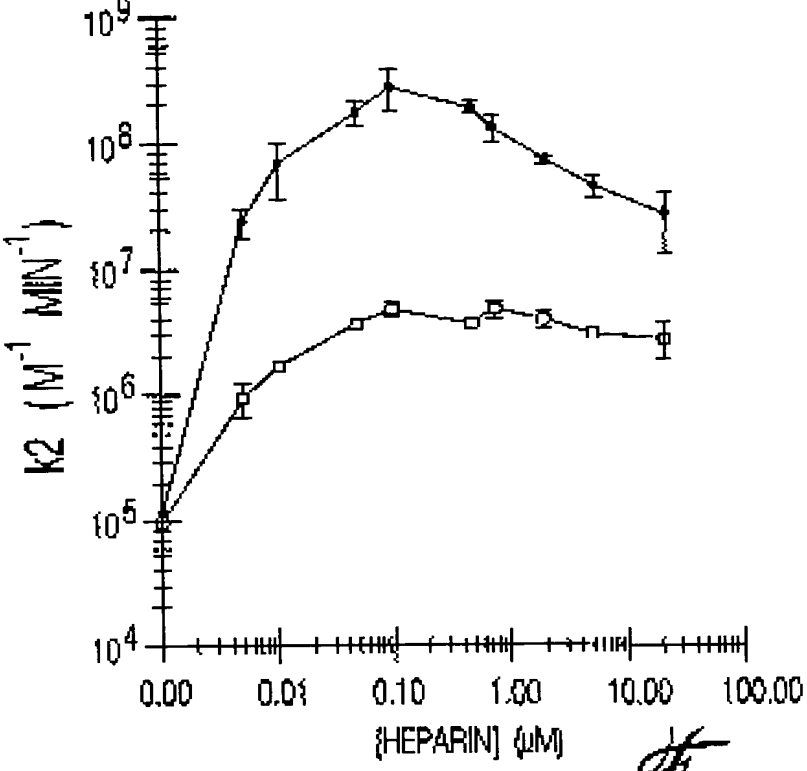

Fig. 6A

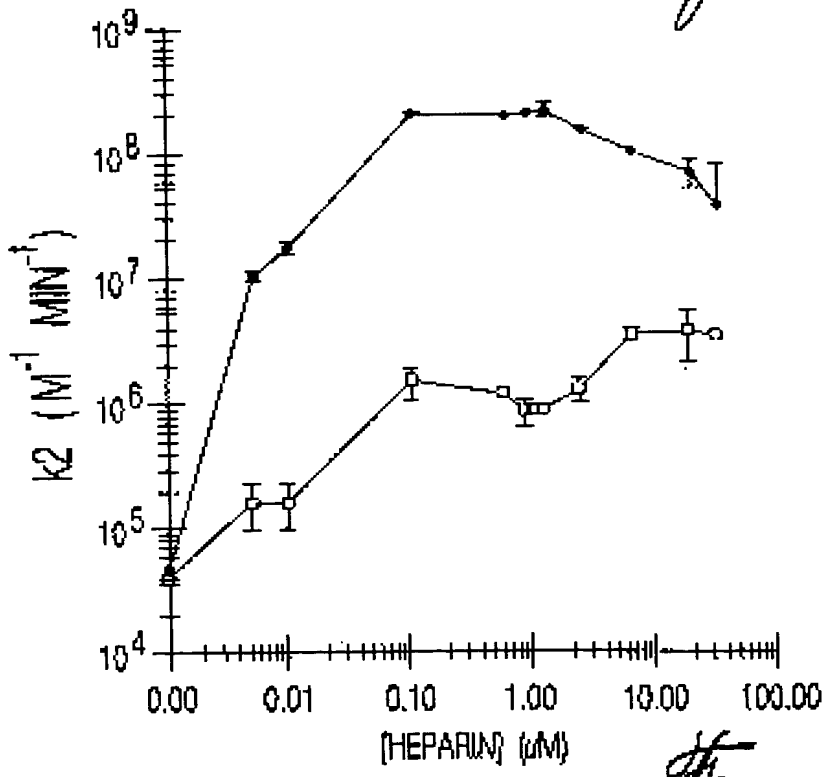

Fig. 6B

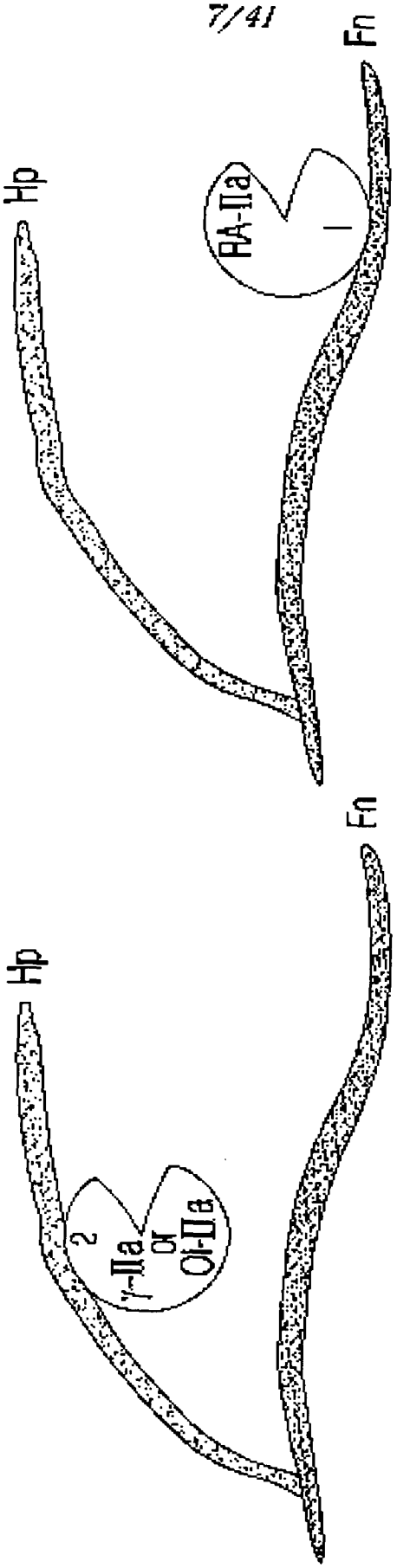

Fig. 1

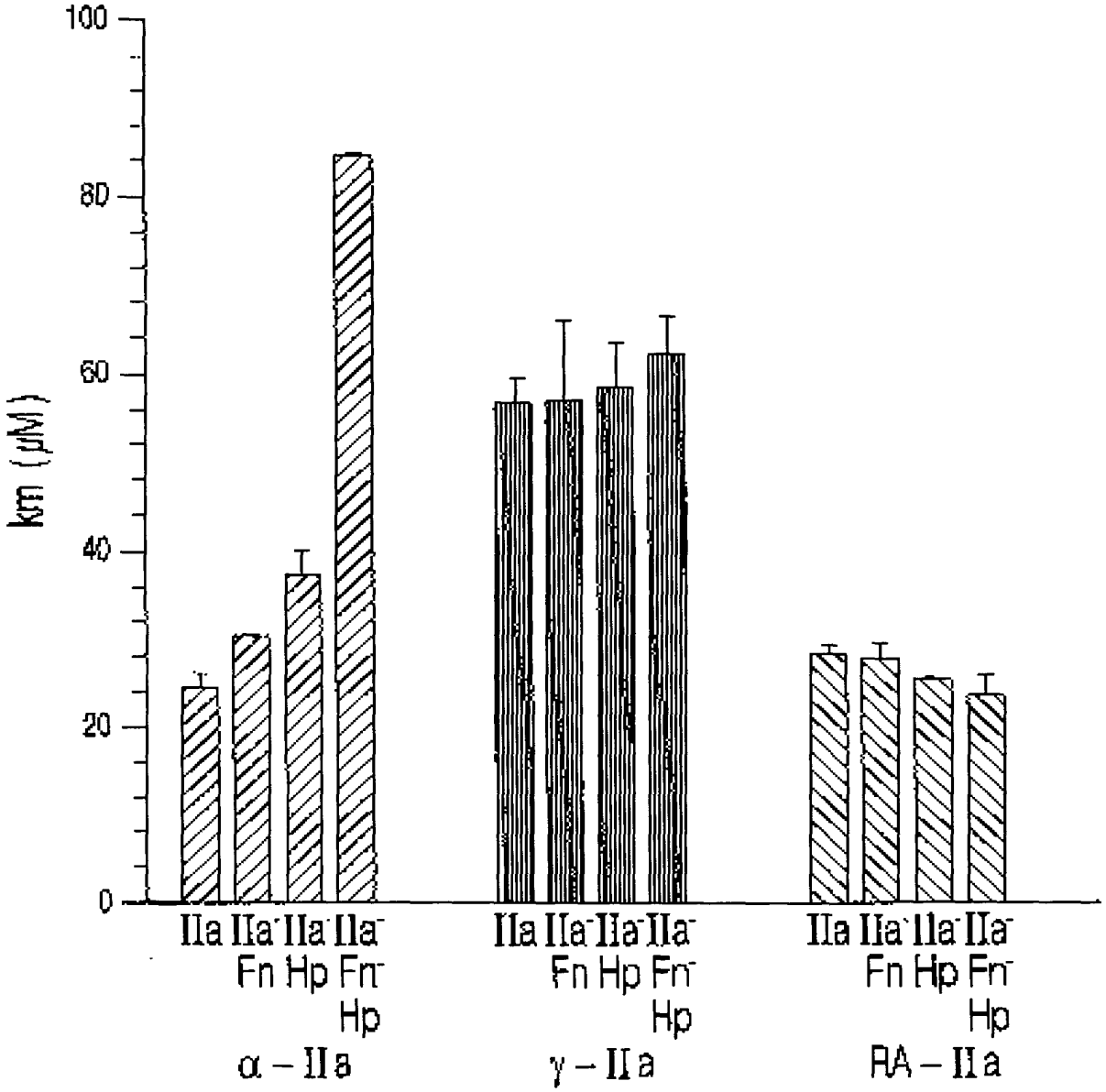

Fig. 8